II β-turns, but neither shows cross-ring bonding.[37] The proline-containing substances *cyclo*(Gly-Pro-XXX)$_2$ (XXX = D-Ala, Gly) exhibit two β-turns but are unsymmetrical and reveal only one intramolecular hydrogen bond in the crystalline state.[38] In all of these cases geometric analysis suggested that unfavorable O···O repulsions induced by the ring conformation prevent close approach of the β-turn N—H/O=C moieties. This effect was predicted earlier by Madison as a consequence of electrostatic repulsion between contiguous cross-ring hydrogen bonds in the cyclic array.[39] Complementary NMR studies, however, have indicated C_2 symmetry in solution where asymmetry exists in the solid.[40] The proline-free hexapeptide *cycl*(Gly-D-Leu-Leu)$_2$ evidences similar properties. Two crystallographically independent molecules are found in the unit cell.[20b] Each possesses two β-turns but only one 4→1 intramolecular hydrogen bond. NMR and CD spectroscopy indicate that one of these most likely equilibrates with yet another conformer in solution. In general, conformational homogeneity in the solid accompanied by conformer averaging in solution is not uncommon.[35] In corroboration, a careful NMR investigation of a series of cyclic hexapeptides led Gierasch to the conclusion that 1,4-hydrogen bonding in the β-turn "is not a strong effect".[41]

These and other studies[42] clearly reveal that β-turns may or may not support hydrogen bonding. When the 4→1 links are manifest, their structural framework can exist side by side both in the crystal and in solution with other conformations that do not require intramolecular hydrogen bonds. The energy difference between the cross-linked and the cross-link-free structures is therefore diminishingly small. Under the circumstances, it is difficult to argue that hydrogen bonding is a serious structural determinant for cyclic hexapeptides. The results for roseotoxin B described above suggest it to be a prototype for the overwhelming importance of ring conformation factors. These, in turn, must be evaluated in terms of torsional freedom, internal dipole interactions, and medium effects.[44] It remains to be seen how structural composition and ring size in cyclic peptides in general partition the various energy components insofar as they contribute to conformational integrity.

Acknowledgment. I thank Prof. Garland Marshall (Washington University School of Medicine, St. Louis) for early access to the MM2 amide parameters and Dr. Jim Springer for providing the roseotoxin B X-ray results prior to publication. Drs. Peter Gund, Tom Halgren, and Bruce Bush (Merck) engaged in stimulating and valuable discussions.

Registry No. 1, 55466-29-0; **4a**, 2503-26-6; **4b**, 27482-48-0; **4c**, 88945-79-3; **4d**, 88945-80-6; **5**, 88945-81-7; **6**, 88945-82-8; **7**, 88945-84-0; **8**, 88945-83-9.

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Systematic Approach to the Analysis of Carbon-13 NMR Spectra of Complex Carbohydrates. 1. α-D-Mannopyranosyl Residues in Oligosaccharides and Their Implications for Studies of Glycoproteins and Glycopeptides

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Abstract: The ^{13}C NMR spectra (at 67.9 MHz) and specific assignments of the ^{13}C resonances are presented for Manα1→2Man, Manα1→2Manα1→2Man, Manα1→2Manα1→2Manα1→2Man, Manα1→3Manα1→2Manα1→2Man, Manα1→4Manα1→0CH$_3$, Manα1→6Manα1→6Man, and the linear (1→6)-α-D-mannopyranan. It is shown that the chemical shifts of all the carbons of a nonreducing α-D-mannopyranose residue are substantially influenced by the nature of the group to which the terminal residue is linked. For example, when going from a Manα1→4Man(α) moiety to Manα1→6Man(α), there are substantial changes in the chemical shifts of all carbons (excpet C-6) of the nonreducing terminal mannose. The α-D-mannosylation of an α- or β-D-mannopyranosyl residue at C-2, C-3, C-4, or C-6 also causes significant changes in the chemical shifts of most carbons of the mannosylated residue. A method is presented for calculating the chemical shifts of any α-D-mannopyranosyl residue linked to other α-D-mannopyranosyl residues (at C-1, C-2, C-3, C-4, or C-6 or any combination of such linkages), on the basis of the chemical shifts of the oligosaccharides listed above. For this purpose, two sets of data are presented: (i) the chemical shifts of nonreducing terminal α-D-mannopyranosyl residues involved in 1→2, 1→3, 1→4, and 1→6 linkages to other α-D-mannopyranosyl residues and (ii) the effects of α-D-mannosylation at C-2, C-3, C-4, and C-6 of an α-D-mannopyranosyl residue. The predictive powers of these two empirical data sets are tested by comparing the experimental and calculated spectra of the α-D-mannopyranosyl residues of Manα1→6(Manα1→3)Manα1→6Manβ1→R, where R = 4GlcNAcβ1→4GlcNAcβ1→Asn. There is very good agreement between the experimental and calculated spectra, to the point that the calculated spectra are readily used to rule out alternate structures such as Manα1→6Manα1→6(Manα1→3)Manβ1→R, on the basis of differences in several regions of the spectrum. It is shown that high-field superconducting NMR spectrometers provide enough resolution in the ^{13}C NMR spectra of complex carbohydrates to allow extensive use of the nonanomeric carbon resonances (and not just those of the anomeric carbons) in structural studies of large oligosaccharides.

The determination of the primary structure of the carbohydrate side chains of glycoproteins is usually a formidable task, both because the available methods are time consuming and often ambiguous and because glycoprotein heterogeneity often greatly

0002-7863/84/1506-2400$01.50/0

increases the difficulties of structure determination. As an illustration, although it has been known since 1963 that bovine pancreatic ribonuclease B contains a single carbohydrate side chain which has about 6 mannose and 2 *N*-acetylglucosamine residues,[1] the heterogeneity of this carbohydrate side chain has only recently been fully recognized[2,3] and the primary structures of its main fractions have only recently been determined.[2-4] It is becoming increasingly apparent that natural-abundance ^{13}C NMR spectroscopy at high magnetic field strengths can play a major role in determinations of the primary structures and other properties of the carbohydrate moieties of glycopeptides[5,6] and even intact glycoproteins.[7,8] This happy situation results in great part from the fact that, at high magnetic field strengths, not only most anomeric carbons but also most of the nonanomeric ones give rise to resolved single-carbon resonances, even in spectra of rather large oligosaccharides.[5,6] However, the realization of the full potential of ^{13}C NMR for this purpose requires an understanding of the effects of glycosidic bond formation on the ^{13}C chemical shifts of the carbohydrate residues. Because of the ubiquitous presence of α-D-mannopyranosyl residues in numerous glycoproteins,[9] it seems particularly desirable, as a first step, to carry out a systematic analysis of the resonances of anomeric and nonanomeric carbons in ^{13}C NMR spectra of α-D-mannopyranosyl oligosaccharides. It is pertinent to quote from a recent special report by Sharon and Lis on glycoproteins in Chemical and Engineering News: *Undoubtedly, when a large enough dictionary of NMR spectra of oligosaccharides and polysaccharides has been built up, other techniques for the structural analysis of such compounds* [glycopeptides] *will become obsolete.*[10]

Gorin has reported the ^{13}C chemical shifts (at 25 MHz) and assignments for the anomeric carbons of various oligosaccharides and polysaccharides composed of α-D-mannopyranosyl residues involved in 1→2, 1→3, and 1→6 linkages.[11a] The resonances of nonanomeric carbons were not analyzed, presumably because of poor spectral resolution at 25 MHz. Ogawa and Sasajima reported the ^{13}C NMR spectra (also at 25 MHz) of various methyl manno oligosaccharides and presented some assignments.[11b] Again, the nonanomeric carbon regions in spectra of trisaccharides and larger oligosaccharides suffered from poor resolution. More recently, Ogawa and Yamamoto reported the ^{13}C NMR spectra (also at 25 MHz) of some linear (1→2)-linked manno oligosaccharides.[11c]

In this paper, we present the ^{13}C chemical shifts at (67.9 MHz) and assignments for the carbons of various α-D-mannopyranosyl oligosaccharides (and one polysaccharide) which contain 1→2, 1→3, 1→4, and 1→6 linkages, together with an analysis of the data which should be helpful in the interpretation of ^{13}C NMR spectra of glycopeptides and glycoproteins. In the following paper in this issue we apply these results to the analysis of the spectra of some compounds of the type $(Man\alpha)_nMan\beta_1\rightarrow$ 4GlcNAcβ1→4GlcNAcβ1→Asn, where n = 3–6, which occur commonly as side chains of glycoproteins.

Perhaps it is pertinent at this point to establish the motivation for using ^{13}C NMR in studies of complex carbohydrates, even though ^{1}H NMR requires a much smaller amount of sample. Our motivation is clearly the enormous resolving power of ^{13}C NMR, as is dramatically illustrated in the following paper.[6] It is safe to predict that ^{1}H and ^{13}C NMR will be applied to different and complementary aspects of complex carbohydrate research.

Table I. Effect of Glycosylation at the Anomeric Carbon on the ^{13}C Chemical Shifts of α-D-Mannopyranose

	chemical shift and effect of glycosylation, ppm[a]					
compd	C-1	C-2	C-3	C-4	C-5	C-6
1a[b]	95.44	72.15	71.70	68.30	73.75	62.41
2[c]	102.14	71.32	72.01	68.19	73.87	62.36
	(+6.70)	(−0.83)	(+0.31)	(−0.11)	(+0.12)	(−0.05)
3[d]	100.74	71.57	72.09	68.29	73.97	62.39
	(+5.30)	(−0.58)	(+0.39)	(−0.01)	(+0.22)	(−0.02)
4[e]	101.77	71.14	71.80	68.15	74.45	62.41
	(+6.33)	(−1.01)	(+0.10)	(−0.15)	(+0.70)	(0)
5[e]	102.77	71.43	71.71	68.20	74.60	62.44
	(+7.33)	(−0.72)	(+0.01)	(−0.10)	(+0.85)	(+0.03)

[a] Each number in parentheses is the difference between the observed chemical shift and the corresponding one of 1a. [b] From Figure 1A. [c] From Figure 1B. [d] 75 mM 3 in H_2O, 33 °C, pH 5.9, with a trace of internal dioxane. [e] Taken from ref 16. About 50 mM compound in H_2O, 40 °C, pH 6.5.

Results and Discussion

We have recorded the ^{13}C NMR spectra of the following compounds: D-Mannose (**1**); methyl α-D-mannopyranoside (**2**); ethyl α-D-mannopyranoside (**3**); O^{γ}-(α-D-mannopyranosyl)-L-serine (**4**); $O^{\gamma 1}$-(α-D-mannopyranosyl)-L-threonine (**5**); Manα1→2Man (**6**); Manα1→2Manα1→2Man (**7**); Manα1→2Manα1→2Manα1→2Man (**8**); Manα1→3Manα1→2Manα1→2Man (9); Manα1→4Manα1→OCH_3 (**10**); Manα1→6Man (**11**); Manα1→6Manα1→6Man (**12**); (1→6)-α-D-mannopyranan (**13**).

In the cases of the compounds which have a reducing terminal mannose residue (**1**, **6**–9, **11**, and **12**), our spectra are those of equilibrium anomeric mixtures of the reducing terminal mannose residues. The signal/noise ratios are adequate only for detection of the two pyranose anomers.[12] We shall use the designations **1a**, **6a**, **7a**, **8a**, **9a**, **11a**, and **12a** for the compounds which contain the α-D-mannopyranose anomer at the reducing end of the oligosaccharides and **1b**, **6b**, **7b**, **8b**, **9b**, **11b**, and **12b** for the corresponding β-pyranose versions of these compounds. For convenience, we shall label the mannose residues M_A, M_B, M_C, etc., starting at the reducing end of each oligosaccharide (at the 4-substituted mannose in the case of **10**). In our notation, M_A1 will refer to C-1 of the reducing mannose residue, etc., and M_A1(**6b**) will refer to M_A1 of compound **6b**, etc.

Consider first the ^{13}C chemical shifts and assignments of the simple monosaccharide derivatives **1a**, and **2**–**5** (Table I). In order to ensure that small chemical shift differences between different compounds are real and not artifacts of slightly different chemical shift referencing procedures, all the chemical shifts of Table I were determined at 67.9 MHz in our laboratory, using the same referencing procedure in each case (see Experimental Section). The assignments for **1**, **2**, **4**, and **5** are taken from the literature,[13-16] and those for **3** are based on the reported ones for **2**.

A comparison of the chemical shifts of C-1 in Table I illustrates two well-established phenomena: (i) The chemical shift of a carbohydrate carbon is strongly influenced (shifted downfield)

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Table II. Chemical Shifts and Assignments for the ^{13}C Resonances of Manα1→2Man (6), Manα1→2Manα1→2Man (7), Manα1→2Manα1→2Manα1→2Man (8), and Manα1→3Manα1→2Manα1→2Man (9)

compd[a]	residue[b]	chemical shift[c] C-1	C-2	C-3	C-4	C-5	C-6
6a	M_A	93.91 (4)	80.38 (5)	71.39 (15)	68.47 (16)	73.77 (11)	62.45 (20)*
	M_B	103.44 (1)	71.39 (15)	71.77 (12)	68.27 (17)	74.54 (9)	62.36 (21)*
6b	M_A	94.79 (3)	79.55 (6)	74.83 (8)	68.15 (18)*	78.00 (7)	62.24 (22)
	M_B	102.70 (2)	71.52 (14)	71.68 (13)	68.06 (19)*	74.18 (10)	
7a	M_A	93.91 (3)	80.54 (4)	71.46 (9)*	68.52 (11)*	73.83 (7)	62.47 (14)*
	M_B	101.81 (2)	79.73 (5)	71.40 (10)*	68.49 (12)*	74.55 (6)	62.47 (14)*
	M_C	103.47 (1)	71.40 (10)[d]	71.83 (8)	68.27 (13)	74.55 (6)	62.41 (15)*
8a	M_A	93.86 (4)	80.64 (5)	71.38 (11)	68.45 (12)	73.78 (9)	62.45 (14)*
	M_B	101.87 (2)*	79.76 (7)	71.38 (11)	68.45 (12)	74.54 (8)	62.45 (14)*
	M_C	101.79 (3)*	79.98 (6)	71.38 (11)	68.45 (12)	74.54 (8)	62.45 (14)*
	M_D	103.48 (1)	71.38 (11)	71.79 (10)	68.23 (13)	74.54 (8)	62.36 (15)*
9a	M_A	93.89 (4)	80.56 (5)	71.37 (13)*	68.47 (15)	73.79 (10)	62.49 (18)*
	M_B	101.80 (3)	79.73 (6)	71.46 (12)*	68.47 (15)	74.60 (9)*	62.49 (18)*
	M_C	103.39 (1)*	70.98 (14)	79.28 (7)	67.57 (17)	74.60 (9)*	62.33 (19)*
	M_D	103.46 (2)*	71.46 (12)[e]	71.82 (11)	68.31 (16)	74.67 (8)*	62.33 (19)*

[a] In each case, a letter a or b following the numerical designation for each compound indicates the α-pyranose or β-pyranose anomer, respectively. [b] In each case, M_A refers to the reducing terminal mannose residue. [c] In ppm downfield from Me_4Si. Sample and spectral conditions are given in the legend of Figure 2. Numbers in parentheses refer to peak designations of Figure 2. *Asterisks* on a group of two or more vertically adjacent chemical shifts indicate that all the corresponding assignments refer to the group as a whole and are not one-to-one assignments. [d] M_C2, M_A3, and M_B3 of 7a have not been assigned on a one-to-one basis. [e] M_D2, M_A3, and M_B3 of 9a have not been assigned on a one-to-one basis.

when that carbon becomes involved in a glycosidic linkage; (ii) the actual magnitude of the downfield shift caused by glycosylation is strongly dependent on the group to which the glycosidic link is formed. However, Table I also illustrates a less widely recognized phenomenon: Although it is well-known that glycosylation at any one carbon effects the chemical shifts of some neighboring carbons,[14] Table I indicates that the *magnitude* of this effect is strongly dependent on the nature of the group to which the glycosidic linkage is formed. For example, the upfield shift of C-2 upon glycosylation of C-1 of α-D-mannopyranose varies from about 0.6 ppm for **3** to about 1.0 ppm for **4**, the downfield shift of C-3 varies from negligible for **5** to about 0.4 ppm for **3**, and the downfield shift of C-5 varies from about 0.1 ppm for **2** to about 0.9 ppm for **5** (Table I).

We shall show that glycosylation at C-1 of an α-D-mannopyranosyl residue by another α-D-mannopyranosyl residue will cause chemical shift variations for most carbons of the former, and that the magnitude of the changes will be influenced by the nature of the linkage (1→2, 1→3, 1→4, or 1→6) to the latter residue. We also present and discuss the effects of α-D-mannosylation at C-2, C-3, C-4, and C-6 of an α-D-mannopyranosyl residue. These results greatly facilitate the analysis of the ^{13}C NMR spectra of glycopeptides and glycoproteins.

For reference purposes in our studies of **6–13**, Figure 1A shows the ^{13}C NMR spectrum of dilute D-mannose (**1**) at anomeric equilibrium in H_2O, and Figure 1B shows the corresponding spectrum of methyl α-D-mannopyranoside (**2**). Figure 2 shows the spectra of the (1→2)-linked compounds **6–8** and also **9** The resonances of C-6 (in the range 62.2–62.5 ppm) are not shown in Figure 2. The chemical shifts and assignments are given in Table II. Consider first the spectrum of the α(1→2)mannobiose **6** (Figure 2A). On the basis of the chemical shifts of **1a** and **1b** (Figure 1A), peak 7 (78.00 ppm) can only arise from M_A5 of **6b** (a β-D-mannopyranose residue), which indicates that all the minor peaks of Figure 2A arise from **6b**, and therefore all the major peaks arise from **6a**. The proportion of the β-anomer is much less in the case of Manα1→2Man than in the case of the monosaccharide (Figure 1A).

It follows from consideration of the chemical shifts of the monosaccharide derivatives (Table I and Figure 1) and from relative intensities that peaks 1, 2, 3, and 4 of Figure 2A arise from M_B1(**6a**), M_B1(**6b**), M_A1(**6b**), and M_A1(**6a**), respectively. It follows from the chemical shifts of M_A1(**6a**) and M_A1(**6b**) (93.91 and 94.79 ppm, respectively) and the corresponding values

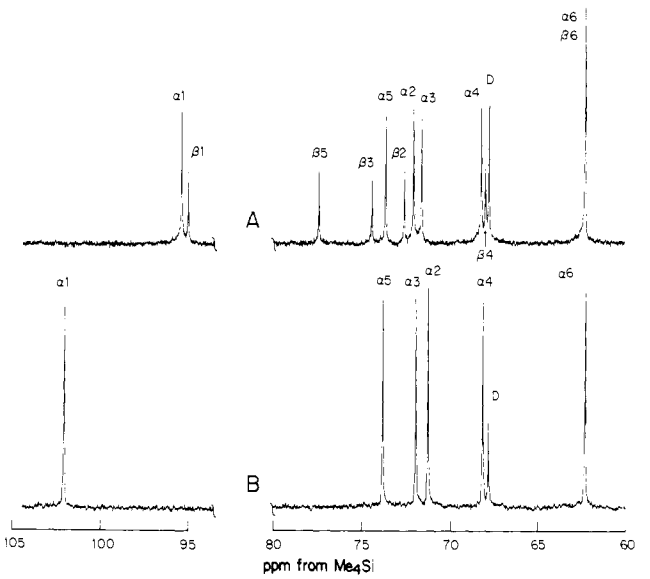

Figure 1. Proton-decoupled ^{13}C NMR spectra of 90 mM **1** (spectrum A) and **2** (spectrum B) in H_2O, recorded at 67.9 MHz with the use of a 15-mm probe, a recycle time of 1.32 s, and 0.5-Hz digital broadening. Other conditions were as follows: pH 6.8 and 2600 scans for **1**, pH 7.8 and 4096 scans for **2**. Specific assignments are shown above each peak, α and β indicate the corresponding pyranose anomers, and the number following α or β indicates the carbon number. The resonance of dioxane is indicated with a D. The chemical shifts of α-D-mannopyranose (**1a**) and those of **2** except for the methyl group are given in Table I. The chemical shifts of β-D-mannopyranose (**1b**) are as follows: 95.06 (C-1), 72.67 (C-2), 74.51 (C-3), 68.06 (C-4), 77.48 (C-5), 62.41 (C-6). The chemical shift of the CH_3 carbon of **2** is 56.02 ppm.

of the anomeric carbons of **1a** and **1b** (95.44 and 95.06 ppm, respectively) that α-D-mannosylation at C-2 of D-mannose causes a large upfield shift for C-1 of the α-pyranose anomer (1.5 ppm) and a relatively small upfield shift for C-1 of the β-pyranose anomer (0.3 ppm). As a consequence, the relative positions of the resonances of C-1 of the α- and β-anomers are reversed when going from free D-mannose (Figure 1A) to the reducing residue (M_A) of **6** (Figure 2A).

The chemical shifts of peak 5 (80.38 ppm) and peak 6 (79.55 ppm) of **6** (Figure 2A) are only consistent with assignments to

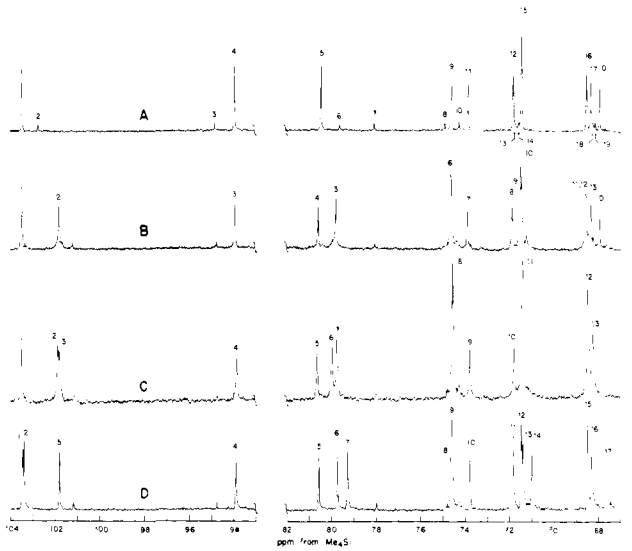

Figure 2. Proton-decoupled ^{13}C NMR spectra (excluding the resonances of C-6) of **6** (A), **7** (B), **8** (C), and **9** (D) in H_2O, recorded at 67.9 MHz with the use of a 15-mm probe (a 10-mm probe for **7**), a recycle time of 1.0 s (1.32 s for **7**), a spectral width of 12 500 Hz (**6** and **9**) or 6250 Hz (**7** and **8**), and 8192 time-domain addresses per channel (4096 for **8**, which was then processed with 8192 addresses per channel by adding a tail of 4096 zeros to each of the two accumulated time-domain blocks). Each spectrum was processed with a digital broadening of 0.0 Hz (**6** and **9**), 0.5 Hz (**7**), or 1.0 Hz (**8**). Sample conditions were as follows: (mM concentration, pH) **6** (90, 7.6), **7** (50, 6.1), **8** (10, 7.0), and **9** (20, 6.7). Peaks are numbered consecutively from left to right. The resonances of the minor β-pyranose form (at the reducing terminal residue) are numbered only in Figure 2A. The resonance of a trace of internal dioxane (present only in the samples of **6** and **7**) is designated D. The chemical shifts of the C-6 resonances, which are upfield of the spectral range shown, are given in Table II.

the glycosidically linked C-2 of M_A (see Figure 1). The relative intensities obviously indicate that peaks 5 and 6 arise from M_A2(**6a**) and M_A2(**6b**), respectively. We have already assigned peak 7 to M_A5(**6b**) (see above). Peaks 9 and 11 must arise from C-5 of the two mannose residues of **6a** (see Table I and Figure 1). The one-to-one assignments of peak 9 (74.54 ppm) and peak 11 (73.77 ppm) to M_B5(**6a**) and M_A5(**6a**), respectively, are based on the following considerations: (i) On the basis of Table I, the chemical shift of C-5 of a nonreducing terminal α-D-mannopyranose residue such as M_B(**6a**) is expected to be ≳74 ppm. (ii) On the other hand, the chemical shift of C-5 of M_A(**6a**) is expected to be ≲73.8 ppm (the value of free α-D-mannopyranose in Table I), because Gorin has reported that C-5 of α-D-mannopyranose undergoes a very slight *upfield* shift (about 0.1 ppm) upon *O*-methylation of C-2.[14]

The small peaks 8 and 10 of Figure 2A can only arise from M_A3 and M_B5 of **6b** (see Figure 1 and Table I). Peak 8 (at 74.83 ppm) is assigned specifically to M_A3(**6b**) because the chemical shift of C-3 of free β-D-mannopyranose is 74.5 ppm (Figure 1A) and *O*-methyl substitution of C-2 of β-D-mannopyranose causes a 0.4 ppm downfield shift of C-3.[14] The chemical shift of peak 10 (74.18 ppm) is within the range observed for C-5 of the α-D-mannopyranosyl residues (glycosylated only at C-1) of Table I. Peak 12 and peak 15 (a two-carbon resonance) of Figure 2A must arise from M_A3(**6a**), M_B2(**6a**), and M_B3(**6a**). The specific assignments of M_B3(**6a**) and M_B2(**6a**) to peak 12 (71.77 ppm) and one-half of peak 15 (71.39 ppm), respectively, are based on the chemical shifts of C-3 and C-2, respectively, of the α-D-mannopyranosyl residues of Table I. The specific assignment of M_A3(**6a**) to one-half of peak 15 follows by elimination and is consistent with the value of 71.75 ppm for C-3 of **1a** (Table I) and the reported *upfield* shift of 0.3 ppm for C-3 of **1a** upon *O*-methylation at C-2 of **1a**.[14] We assign peaks 13 and 14 of Figure 2A to M_B3(**6b**) and M_B2(**6b**), respectively, on the basis of the data for α-D-mannopyranosyl residues in Table I. The weak resonances at 71.68 and 71.52 ppm (peaks 13 and 14, respectively) are assigned to M_B3(**6b**) and M_B2(**6b**), respectively, on the basis of a comparison of their chemical shifts with those of C-3 and C-2 of the α-D-mannopyranosyl residues in Table I.

On the basis of their relative intensities and the chemical shifts of Table I, peaks 16 and 17 of Figure 2A must arise from M_A4 and M_B4 of **6a** and peaks 18 and 19 must arise from M_A4 and M_B4 of **6b**. The one-to-one assignments of peak 16 (68.47 ppm) and peak 17 (68.27 ppm) to M_A4(**6a**) and M_B4(**6a**), respectively, come from the following evidence: (i) The chemical shift of C-4 of free α-D-mannopyranose is 68.30 ppm (Table I). (ii) Table I indicates that glycosylation at C-1 of α-D-mannopyranose should either cause a negligible change in the chemical shift of C-4 or cause a slight *upfield* shift. (iii) Methylation at C-2 of α-D-mannopyranose causes a slight *downfield* shift of C-4.[14] We do not have one-to-one assignments for M_A4(**6b**) and M_B4(**6b**).

We assign the resonances at 62.45 and 62.36 ppm (not shown in the range of chemical shifts of Figure 2A) to M_A6 and M_B6 of **6a**, but not on a one-to-one basis. A small resonance at 62.24 ppm is assigned to M_A6 or M_B6 of **6b**; one or the other of these carbons must contribute an unresolved weak component within the range of the strong resonances at 62.45 and 62.36 ppm.

Consider now the spectrum of the α(1→2)mannotriose **7** (Figure 2B). The chemical shifts and assignments of **7a** are given in Table II. The assignments follow readily from those of **6**. First, since **7** is derived from **6** by having an extra α-D-mannopyranosyl residue attached to C-2 of M_B(**6**), it is obvious that the chemical shifts of M_A(**7a**) and M_A(**7b**) should be indistinguishable from those of M_A(**6a**) and M_A(**6b**), respectively. On this basis, peak 3 (93.91 ppm), peak 4 (80.54 ppm), and peak 7 (73.83 ppm) of Figure 2B are assigned to C-1, C-2, and C-5, respectively, of M_A(**7a**). Also, M_A3(**7a**) is assigned to peak 9 (71.46 ppm) or one-half of peak 10 (a two-carbon resonance at 71.40 ppm), and M_A4(**7a**) gives rise to peak 11 (68.52 ppm) or peak 12 (68.49 ppm). The small resonances at 94.75, 78.00, and 74.82 ppm (which are not designated with numbers in Figure 2B) must be assigned to C-1, C-5, and C-3, respectively, of M_A(**7b**). The resonances of C-2 and C-4 of M_A(**7b**) are not clearly observable within the signal/noise ratio of Figure 2B; we expect them at about 79.6 and 68.1 ppm (on the basis of M_A of **6b**), very close to the strong peaks 5 and 13, respectively, of Figure 2B.

By elimination and from consideration of the chemical shifts of Table I, peaks 1 and 2 of Figure 2B must be assigned to C-1 of M_B(**7a**) and M_C(**7a**), but not on a one-to-one basis. The specific assignments of peak 1 (103.47 ppm) and peak 2 (101.81 ppm) to M_C1(**7a**) and M_B1(**7a**), respectively, are based on the observation that M_B1(**6a**) resonates at 103.47 ppm (Table II) and that the resonance of C-1 of an α-D-mannopyranosyl residue is expected to move about 1.5 ppm upfield upon α-D-mannosylation of C-2.[11] Also by elimination and from consideration of the chemical shifts of Table I, peak 5 (79.73 ppm) must be assigned to the α-D-mannosylated C-2 of M_B(**7a**), and peak 6 (a two-carbon resonance at 74.55 ppm) must arise from C-5 of M_B(**7a**) and M_C(**7a**).

We have already seen that M_A3(**7a**) gives rise to peak 9 (71.46 ppm) or one-half of peak 10 (71.40 ppm). The other contributors in the range of peaks 8–10 must be M_B3(**7a**), M_C2(**7**), and M_C3(**7**). Our specific assignment of peak 8 (71.83 ppm) to M_C3(**7**) is based on the observation that the nonreducing α-D-mannopyranosyl residues of Table I consistently show C-3 about 0.3–0.7 ppm *downfield* of C-2. Independent evidence for our specific assignment of peak 8 to M_C3(**7**) comes from Gorin's report that C-3 of α-D-mannopyranose undergoes a 0.3 ppm *upfield* shift upon *O*-methylation of C-2,[14] because our assignment implies that M_B3(**7a**), at 71.46 or 71.40 ppm, resonates 0.31 or 0.37 ppm upfield of M_B3(**6a**) (Table II). Peak 13 (68.27 ppm) and either peak 11 (68.52 ppm) or peak 12 (68.49 ppm) must arise from M_B4(**7a**) and M_C4(**7**). Our specific assignment of peak 13 to M_C4(**7**) is basedon Gorin's report that C-4 of α-D-mannopyranose moves 0.3 ppm *downfield* upon *O*-methylation of C-2,[14] because our assignment implies that M_B4(**7a**), at 68.52 or 68.49 ppm, resonates 0.25 or 0.22 ppm downfield of M_B4(**6a**) (Table II).

The assignments for the α(1→2)mannotetraose **8** (Figure 2C and Table II) follow readily from those of **6** and **7**.

Consider now the spectrum of Manα1→3Manα1→2Manα1→2Man (**9**) (Figure 2D and Table II). Our assignments for M_A and M_B of **9a** and **9b** are based on the very safe assumption that the chemical shifts of these residues should be essentially identical with the corresponding ones of M_A and M_B of **7a** and **7b** and of **8a** and **8b**. The assignments for M_C and M_D of **9** (Figure 2D) follow readily by elimination and from considerations of chemical shifts, as follows. Because peak 3 (101.80 ppm) is assigned to M_B1 of **9a**, peaks 1 and 2 must be assigned to M_C1 and M_D1 of 9 (not on a one-to-one basis). The chemical shifts of these resonances (103.46 and 103.39 ppm) are consistent with assignments to α-D-mannopyranosyl residues which are unglycosylated at C-2 and whode anomeric carbons are linked to C-2 or C-3 of other α-D-mannopyranosyl residues.[11] Because peak 5 (80.56 ppm) and peak 6 (79.73 ppm) are assigned to M_A2 and M_B2 of 9**a**, peak 7 is assigned to M_C3 of **9**. The chemical shift of peak 7 (79.28 ppm) is consistent with an assignment to a glycosylated C-3 of an α-D-mannopyranosyl residue.[11]

By elimination, peak 11, a one-carbon contributor to peaks 12–13, and peak 14 (Figure 2D) must arise from M_C2, M_D2, and M_D3. The specific assignments of peak 11 (71.82 ppm) and of one component of peaks 12–13 (71.46 or 71.37 ppm) to M_D3 and M_D2, respectively, follow from the data of Table I. The specific assignment of peak 14 (70.98 ppm) to M_C2 is consistent with the expected upfield shift of C-2 upon glycosylation of C-3 of an α-D-mannopyranosyl residue.[14] By elimination, peaks 16 and 17 must arise from M_C4 and M_D4. The specific assignment of peak 16 (68.31 ppm) to M_D4 follows from Table I. The assignment of peak 17 (67.57 ppm) to M_C4 is consistent with the expected upfield shift of C-4 upon glycosylation of C-3 of an α-D-mannopyranose residue.[14]

All of the above assignments for **6**, **7**, **8** and **9** (Table II) rely on evidence based on (i) the reported chemical shifts and assignments for **1**, **2**, and related compounds (Table I), (ii) reported chemical shift changes caused by O-methylation of C-1, C-2, and C-3 of D-mannose,[14] and (iii) internal comparison when going from **6** to **7** to **8** to 9. Independent supporting evidence for some of the one-to-one assignments can be obtained from the use of comprisons of chemical shifts of samples in H_2O and D_2O, because substitution of a hydroxyl hydrogen by deuterium causes an upfield shift of about 0.1 ppm of the resonance of the carbon which bears the hydroxyl group (β effect) and a much smaller upfield shift (≲0.03 ppm) on a carbon bonded to the carbon bearing the hydroxyl group (γ effect).[17-19] The β effect is particularly useful for assigning the resonances of C-5 of α-D-mannopyranose residues glycosylated at C-4 or C-6, because in both of these cases the resonance of C-5 (which does not bear a hydroxyl hydrogen and, therefore, lacks a β effect) moves from its normal range of about 74–75 ppm (Table I) upfield into the range of resonances of C-2 and C-3. Also, we find that γ effects are useful for identifying the resonances of C-3 of mannose residues glycosylated at C-1 but not at C-2 and C-4, because C-3 of such a residue is the only carbon subject to one β effect plus two γ effects.

Consider first the deuterium isotope effects for **1a**, **1b**, and **2** (Table III). Here Δ is the chemical shift in H_2O minus the corresponding one in D_2O. The carbons which lack β effects (C-5 of all three structures and C-1 of **2**) have Δ ≲ 0.04 ppm. The values for C-5 (about 0.03 ppm) are larger than those of C-1 of **2**, which is consistent with the fact that C-5 has two γ effects (from C-4 and C-6) in all three structures, while C-1 of **2** has only one γ effect (from C-2). For carbons with a β effect (directly bonded OH group), we find that, as expected, Δ ≳ 0.1 ppm. The Δ values for carbons with one β and two γ effects (C-3 of **2** and C-2 and C-3 of **1a** and **1b**) are about 0.15–0.16 ppm, while Δ values for carbons with one β and one γ effect are typically in the range of 0.110–0.125 ppm.

Table III. Changes in the ^{13}C Chemical Shifts of D-Mannose and Derivatives When Going from H_2O to D_2O Solution

compd[a]	residue[b]	Δ, ppb[c] C-1	C-2	C-3	C-4	C-5	C-6
1a		110	160	160	126	39	118
1b		96	152	157	126	28	118
2		3	115	154	115	31	110
6a	M_B	−8	124	157	118	20	118*
	M_A	87	14	124	132	31	107*
7a	M_C	−3	120[d]	154	121	25	112
	M_B	−11	−11	120[d]	130[d]	25	112
	M_A	90	17	130[d]	140[d]	37	112
9a	M_D	−6*	120[d]	163	124	34*	118*
	M_C	11*	90	84	73	28*	118*
	M_B	−22	−17	130[d]	124*	34*	112*
	M_A	96	11	130[d]	140*	39	107*
10	M_B	−3	135	160	120[d]	37[e]	112*
	M_A	8	124	118	8[e]	20	124*
11a	M_B	11	121	155	121	34	115
	M_A	110	160	149	124	20	8
11b	M_B	11	120[d]	160[d]	120[d]	31	[f]
	M_A	98	155	146	108	17	6
13		3	124	152	112	11	−11

[a] See footnote *a* of Table II. [b] See footnote *b* of Table II. [c] Δ is expressed in parts per *billion*. Each value was obtained by subtracting a chemical shift of the compound in D_2O from the corresponding one in H_2O. Sample and spectral conditions are essentially those of Figures 1–4. See Experimental Section for other details. Values within a group of two or more vertically adjacent ones labeled with *asterisks* have not been assigned on a one-to-one basis within the group, because of a lack of one-to-one assignments for the corresponding resonances (see Tables II and IV). Unless otherwise indicated, each Δ value has a precision of about ±7 ppb. [d] Estimated precision is about ±15 ppb because of peak overlap or ambiguity of assignments of closely spaced resonances, or both. [e] M_A4 and M_B5 of **10** have not been assigned on a one-to-one basis. [f] This resonance of the minor β-pyranose anomer is not resolved enough from that of the corresponding one of the major α-pyranose anomer to allow a measurement of Δ.

Our Δ values for **6**, **7**, and **9** (Table III) confirm many of the assignments of Table II. The assignments for C-5 are confirmed from the Δ values of about 0.025–0.040 ppm. The *relative* assignments of the unglycosylated C-2 and C-3 (closely spaced resonances) of M_B of **6a**, M_C of **7a**, and M_D of **9a** are confirmed because the Δ values are about 0.15–0.16 ppm for C-3 and about 0.12 ppm for C-2. The specific assignment for M_C3(**9a**) (peak 7 of Figure 2D), relative to those of M_A2(**9a**) and M_B2(**9a**), is confirmed by its relatively large Δ value (for a carbon without a free hydroxyl group) of 0.08 ppm, compared to 0.01 and −0.02 ppm for M_A2 and M_B2, respectively. The large Δ value of M_C3 (for a glycosylated carbon) is consistent with the fact that this is the only carbon of 9**a** which has two γ effects. In fact, the Δ value of M_C3 is similar to those of unglycosylated carbons (β effect) which lack γ effects.

Unkefer and Gander have reported the chemical shifts (at 25 MHz) and tentative assignments for the ^{13}C resonances of **6a** and **7a**.[20] In each case, our assignments for C-2 and C-3 of the nonreducing terminal residue are the reverse of theirs, and our assignments for C-4 of the reducing and nonreducing terminal residues are also reversed relative to those of Unkefer and Gander.[20]

Consider now the spectrum of Manα1→4Manα1→OCH_3 (**10**), shown in Figure 3. Chemical shifts and assignments are given in Table IV. The specific assignment for M_A1 (peak 2 at 102.0 ppm) follows from the observation that C-1 of methyl α-D-mannopyranoside is at 102.14 ppm (Table I) and that C-1 of α-D-mannopyranose undergoes a 0.1 ppm *upfield* shift upon

(17) Ho, S.-C.; Koch, H. J.; Stuart, R. S. *Carbohydr. Res.* **1978**, *64*, 251–256.
(18) Pfeffer, P. E.; Valentine, K. M.; Parrish, F. W. *J. Am. Chem. Soc.* **1979**, *101*, 1265–1274.
(19) Pfeffer, P. E.; Parrish, F. W.; Unruh, J. *Carbohydr. Res.* **1980**, *84*, 13–23.
(20) Unkefer, C. J.; Gander, J. E. *J. Biol. Chem.* **1979**, *254*, 12131–12135. About 1.4 ppm should be subtracted from each chemical shift of this publication in order to convert to our chemical shift referencing system.

Table IV. Chemical Shifts and Assignments for the ^{13}C Resonances of Manα1→4Manα1→OCH_3 (**10**), Manα1→6Man (**11**), Manα1→6Manα1→6Man (**12**), and the Unbranched (1→6)-α-D-Mannopyranan (**13**)

compd[a]	residue[b]	chemical shift[c]					
		C-1	C-2	C-3	C-4	C-5	C-6
10	M_A	102.00 (2)	71.81 (7)	72.43 (5)	75.55 (3)[d]	72.37 (5)	62.46 (10)*
	M_B	102.74 (1)	71.69 (8)	71.81 (7)	68.00 (9)	75.03 (4)[d]	62.34 (11)*
11a	M_A	95.58 (3)	72.13 (10)	71.91 (13)	68.14 (16)	71.96 (12)	67.16 (19)
	M_B	100.86 (2)	71.35 (14)	72.01 (11)	68.18 (15)	74.04 (7)	62.36 (20)
11b	M_A	95.22 (4)	72.61 (9)	74.63 (6)	67.93 (17)	75.60 (5)	67.21 (18)
	M_B	100.88 (1)	71.3[e]	72.0[e]	68.2[e]	74.00 (8)	62.4[e]
12a	M_A	95.61 (4)	72.19 (11)	71.97 (13)	68.14 (18)	71.89 (14)*	67.04 (22)
	M_B	100.84 (2)	71.32 (16)	72.23 (10)	68.08 (19)	72.04 (12)*	66.95 (23)
	M_C	100.63 (3)	71.42 (15)	72.04 (12)	68.21 (17)	74.07 (8)	62.37 (24)
12b	M_A	95.25 (5)	72.64 (9)	74.66 (7)	67.93 (20)	75.52 (6)	67.11 (21)
13		100.65 (1)	71.41 (4)	72.29 (2)	68.04 (5)	72.02 (3)	66.83 (6)

[a] A letter a or b following a compound designation indicates the α-pyranose or β-pyranose anomer at the reducing end of the compound. [b] For compound 10, M_A is the α-D-mannopyranose residue linked to the methyl group. For the other compounds, M_A is the reducing terminal residue. [c] In ppm downfield from Me_4Si. Sample and spectral conditions are given in the legends of Figures 3 and 4. Numbers in parentheses refer to peak designations in Figures 3 and 4. *Asterisks* on two vertically adjacent chemical shifts indicate that the corresponding assignments are not one-to-one assignments. [d] M_A4 and M_B5 of **10** have not been assigned on a one-to-one basis. [e] Shoulder on the corresponding resonance of **11a**. Although this resonance is not clearly discernible in Figure 4B, its position was established from a series of spectra of **11** in H_2O, D_2O, and a mixture of the two solvents.

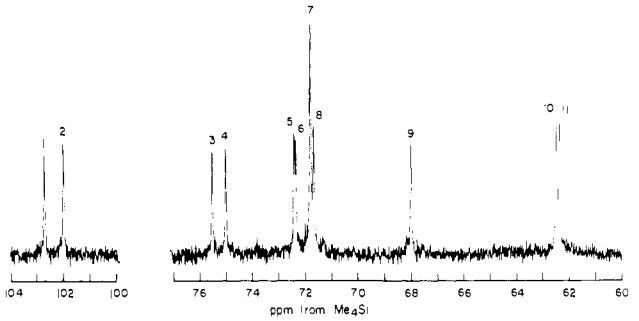

Figure 3. Proton-decoupled ^{13}C NMR spectrum of 13 mM **10** in H_2O (pH 6.3), recorded at 67.9 MHz in a 10-mm probe, with a spectral width of 6250 Hz, 4096 time-domain addresses per channel (processed with 8192 addresses per channel), a recycle time of 1.32 s, and 28 000 scans. No digital broadening was applied. The resonance of the methyl group is at 56.05 ppm.

O-methylation of C-4.[13] By elimination, peak 1 (102.74 ppm) is assigned to M_B1. We assign peak 3 (75.55 ppm) and peak 4 (75.03 ppm) to M_B5 and M_A4, but not on a one-to-one basis, from the following evidence: (i) M_B5 is expected in this region (see Tables I and II). (ii) On the basis of the reported effect of *O*-methylation of C-4 of α-D-mannopyranose on the chemical shift of C-5,[13] we expect an *upfield* shift of about 1 ppm when going from C-5 of **2** (73.87 ppm in Table I) to M_A5(**10**); thus, we expect to find M_A5(**10**) at about 73 ppm. (iii) The effect of glycosylation of C-4 when going from **2** to M_A(**10**) should cause a large downfield displacement of the resonance of C-4. The above evidence does not yield one-to-one assignments for M_A4 and M_B5. Deuterium isotope effects do not yield one-to-one assignments either, because each of these unglycosylated carbons has one γ effect.

Clearly, peaks 5–8 of Figure 3 (in the range 71.7–72.4 ppm) must arise from M_A2, M_A3, M_A5, M_B2, and M_B3 (peak 7 is a two-carbon resonance) of **10**. All of these carbons except M_A5 have free hydroxyl groups. Therefore, the Δ value of 0.02 ppm of peak 6 indicates that this is the resonance of M_A5. Also, M_B3 is the only one of these carbons with one β and two γ effects and should therefore have the largest Δ value. One contributor to peak 7 (which is a two-carbon resonance at 71.81 ppm) is assigned to M_B3 because it has Δ = 0.16 ppm. By elimination, peak 5, one-half of peak 7, and peak 8 must arise from M_A2, M_A3, and M_B2. The specific assignment of M_B2 (peak 8 at 71.69 ppm) is based on the observation that an unglycosylated C-2 of a non reducing α-D-mannopyranosyl residue is expected upfield of an

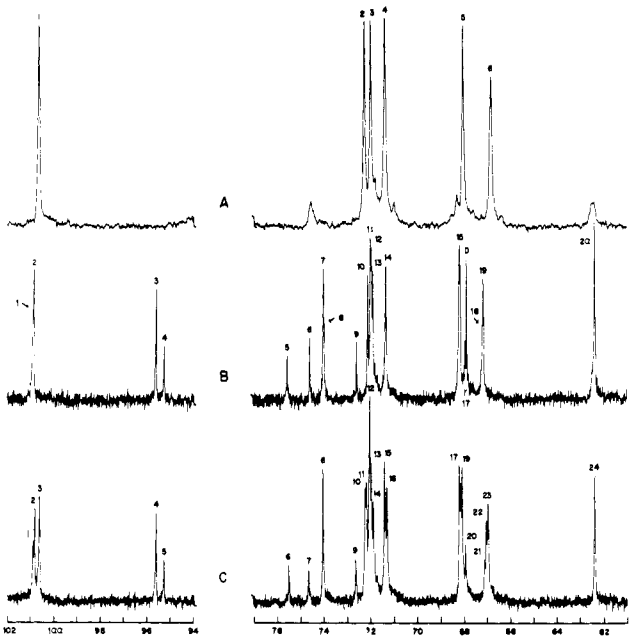

Figure 4. Proton-decoupled ^{13}C NMR spectra of **13** (A), **11** (B), and **12** (C) in H_2O, recorded at 67.9 MHz in a 15-mm (**13**, **12**) or 10-mm probe (**11**). Other conditions were as follows: (concentration, pH, number of scans, digital broadening in hertz, spectral width in hertz, recycle time in seconds) **13** (25 mg/mL, 4.1, 10 000, 1.0, 12 500, 1.0), **11** (30 mM, 6.9, 14 300, 0.1, 6250, 1.32), and **12** (50 mM, not recorded, 7600, 0, 5000, 1.65). Peaks are numbered consecutively from left to right. Peaks 1, 8, and 18 of Figure 2B are shoulders which are better resolved in spectra with greater digital resolution (see Experimental Section). The resonance of internal dioxane (Figure 2B) is labeled D.

unglycosylated C-3 (Tables I and II). Therefore, M_B2 is expected upfield of 71.81 ppm (M_B3). The specific assignment of peak 5 (72.43 ppm) and one-half of peak 7 (71.81 ppm) to M_A3 and M_A2, respectively, is based on the observation that an unglycosylated C-2 should resonate *downfield* of an unglycosylated C-3 in the case of a *reducing* α-D-mannopyranosyl residue (Table I).

Peak 9 of Figure 3 (at 68.00 ppm) must arise from M_B4 of **10**, and peaks 10 and 11 must be the two C-6 resonances (see Table I). The methyl carbon is at 56.05 ppm.

Consider now the (1→6)-linked oligosaccharides Manα1→6Man (**11**) and Manα1→6Manα1→6Man (**12**), and the un-

branched (1→6)-α-D-mannopyranan (**13**), whose spectra are shown in Figure 4, and whose chemical shifts are given in Table IV. It is convenient to examine first the spectrum of **13** (Figure 4A). The assignments of peak 1 (100.65 ppm) and peak 5 (68.04 ppm) to C-1 and C-4, respectively, follow from the data of Table I. Peaks 3 and 6 have Δ values ≲0.01 ppm while all other nonanomeric carbon resonances have Δ > 0.1 ppm (Table III). Therefore, peaks 3 and 6 must arise from C-5 and C-6. The specific assignments of peak 3 (72.02 ppm) and peak 6 (66.83 ppm) to C-5 and C-6, respectively, follow from chemical shift considerations, as follows. Peak 3 is about 2 ppm upfield from C-5 and about 10 ppm downfield from C-6 of alkyl α-D-mannopyranosides, while peak 6 is about 7 ppm upfield of C-5 and about 4.5 ppm downfield of C-6 of alkyl α-D-mannopyranosides (Table I). The assignment of peaks 3 and 6 to C-6 and C-5, respectively, would imply that upon glycosylation at C-6 there is a 10 ppm downfield shift of C-6 and a 7 ppm upfield shift of C-5. The latter effect is unreasonable.[14] On the other hand, the reverse specific assignments would imply a 4.5 ppm downfield shift of C-6 and a 2 ppm upfield shift of C-5, which is consistent with published chemical shift changes upon glycosylation of α-D-mannopyranose.[14] Furthermore, the specific assignment of peak 6 to the only methylene carbon is consistent with the greater line width of peak 6 relative to peaks 1–5.[21] Finally, the relative assignments of the resonances of C-5 and C-6 are confirmed (although marginally so) from their Δ values of 0.01 and −0.01 ppm, respectively. Note that C-5 of **13** has one γ effect while C-6 has none.

Consider now the spectrum of Manα1→6Man (**11**), shown in Figure 4B. Peak 5 (75.60 ppm) and peak 6 (74.63 ppm) can only arise from M_A5 and M_A3 of **11b**, because in the spectrum of free mannose (Figure 1A) the only nonanomeric carbon resonances which have chemical shifts downfield of 74 ppm are C-3 and C-5 of β-D-mannopyranose (at 74.51 and 77.48 ppm, respectively). We assign peaks 5 and 6 to C-5 and C-3, respectively, on the basis of chemical shifts: the chemical shift of C-3 should be fairly invariant when going from **1b** to M_A(**11b**), while the resonance of C-5 is expected to undergo an upfield shift upon glycosylation at C-6.[14] The relative assignments of peaks 5 and 6 are confirmed from their Δ values of 0.02 and 0.15 ppm, respectively (Table III).

The above assignments for peaks 5 and 6 establish that all the small resonances of Figure 4B arise from **11b** and not **11a**. The proportion of the β anomer (at the reducing end) is much greater in the cases of the (1→6)-linked compounds of Figure 4B,C than the (1→2)-linked ones of Figure 2 but still not as great as in aqueous free mannose (Figure 1A).

Peaks 1 and 2 in the spectrum of **11** (Figure 4B) must arise from the anomeric carbons of M_B of **11b** and **11a**, respectively. Peaks 3 and 4 must arise from the anomeric carbons of M_A of **11a** and **11b**, respectively. Peaks 7 and 8 (at 74.04 and 74.00 ppm) can only arise from M_B5 of **11a** and **11b**, respectively (see chemical shifts of Table III). We rule out the presence of M_A5(**11a**) in this region because of the safe expectation that C-5 undergoes a major upfield shift when going from **1a** to M_A(**11a**), as evidenced by the fact that the chemical shift of C-5 of **13** is 72.02 ppm (see above).

Peak 9 of Figure 4B (72.61 ppm) is assigned to M_A2(**11b**), because it is one of the small resonances and because the chemical shift of C-2(**1b**) is 72.67 ppm.

The region of peaks 10–14 of Figure 4B must contain the resonances of C-2 and C-3 of both residues of **11a** and of M_B of **11b** (see Table I). This region should also contain the resonance of M_A5 of **11a** (see above). On the basis of its Δ value of 0.02 ppm, peak 12 (at 71.96 ppm) is assigned to M_A5 of **11a**. We have seen above that the chemical shift of M_A2 of **11b** is 72.61 ppm, which differs by only about 0.05 ppm from that of C-2 of β-D-mannopyranose (Figure 1A). Thus, glycosylation at C-6 does not appreciably change the chemical shift of C-2 of β-D-mannopyranose, and we can safely assume that this is also the case for α-D-mannopyranose. On this basis, peak 10 of Figure 4B (at 72.13 ppm) is assigned to M_A2 of **11a**, because the chemical shift of C-2 of α-D-mannopyranose is 72.15 ppm (Table I).

By elimination, peaks 11, 13, and 14 of Figure 4B must arise from M_A3, M_B2, and M_B3 of **11a** (large resonances) and from M_B2 and M_B3 of **11b** (minor resonances). The resonances of M_B of **11b** are expected to be very close to the corresponding ones of **11a**. Peak 14, which has an upfield shoulder not clearly discernible in Figure 4B, has a Δ value of 0.12 ppm, while peaks 11 and 13 have Δ values of 0.16 and 0.15 ppm, respectively. Therefore, peak 14 (71.35 ppm) and its upfield shoulder are assigned to M_B2 of **11a** and **11b**, respectively. Peak 11 has a discernible upfield shoulder while peak 13 is symmetric. On this basis, peak 11 (72.01 ppm) and its upfield shoulder are assigned to M_B3 of **11a** and **11b**, respectively, and peak 13 (71.91 ppm) is assigned to M_A3 of **11a**.

On the basis of their chemical shifts and relative intensities, peak 15 (68.18 ppm), peak 16 (68.14 ppm), and peak 17 (67.93 ppm) are assigned to M_B4 of **11a** and **11b**, M_A4 of **11a**, and M_A4 of **11b**, respectively. The assignments for **13** (Table IV) and relative intensities indicate that peak 18 (67.21 ppm) and peak 19 (67.17 ppm) must arise from M_A6 of **11b** and **11a**, respectively. Peak 20 (62.36 ppm) must be assigned to M_B6 of **11a** and **11b**.

Consider now the spectrum of Manα1→6Manα1→6Man (**12**) shown in Figure 4C. The assignment of the resonances of M_A of **12a** and **12b** is relatively simple, if we consider that their chemical shifts must be essentially identical with the corresponding ones of M_A(**11a**) and M_A(**11b**), respectively (Table IV). On this basis, we assign peaks 5, 6, 7, 9, 20, and 21 to C-1, C-5, C-3, C-2, C-4, and C-6 of M_A(**12b**), and we assign peak 4 to C-1 of M_A-(**12a**). The other resonances of M_A(**12a**) cannot be identified simply by using the chemical shifts of M_A(**11a**), because they are too close to resonances of M_B(**12a**) or M_C(**12a**) (see below).

In the region of anomeric carbon resonances of M_B and M_C of **12** (peaks 1–3 of Figure 4C), we expect M_B1(**12b**) to be slightly resolved from M_B1(**12a**), in analogy to the resonances of M_B1(**11b**) and M_B1(**11a**), and we expect that the resonances of M_C1(**12a**) and M_C1(**12b**) will coincide, because M_C is far removed from the reducing residue M_A. On this basis (and relative intensities), we assign peak 1 (100.88 ppm) and peak 2 to M_B1(**12b**) and M_B1-(**12a**), respectively, and we assign peak 3 (100.63 ppm) to M_C1 of **12a** and **12b**. The chemical shift of peak 8 (74.07 ppm) is consistent only with an assignment to C-5 of the nonreducing terminal residue M_C (see Table I), because M_B5 is expected in the vicinity of 72 ppm (shifted about 2 ppm upfield by the α-D-mannosylation at M_B6), in analogy to the chemical shift of C-5 of **13**.

The range of peaks 10–16 (72.2 to 71.3 ppm) of Figure 4C must contain the resonances of C-2 and C-3 of all three residues of **12a** (see Table I), as well as those of M_A5(**12a**) and M_B5(**12a**). The task of making specific assignments is made difficult by the crowded nature of this spectral region. However, with the use of spectra in H_2O, D_2O, and an equimolar mixture of the two solvents, we were able to determine that peak 14 and one-half of peak 12 have negligible Δ values, while the other resonances in this region have Δ ≳ 0.12 ppm. On this basis, we assign peak 14 (71.89 ppm) and one-half of peak 12 (72.04 ppm) to M_A5(**12a**) and M_B5(**12a**), but not on a one-to-one basis. The chemical shift of M_A5(**11a**) is about half way between 71.89 and 72.04 ppm (Table IV), and therefore does not resolve the ambiguity. The remaining resonances in the range of peaks 10–16 of Figure 4C must arise from C-2 and C-3 of all three residues of **12a**. Four of these carbons (C-3 of all three residues and C-2 of the reducing terminal residue) each have one β-hydrogen and two γ-hydrogens and should therefore exhibit Δ ≈ 0.15 ppm, while C-2 of M_B and M_C have one less γ-hydrogen and should show Δ ≈ 0.12 ppm. The region of peaks 10–14 is too crowded (especially when D_2O is the solvent) to distinguish 0.12 from 0.15 ppm changes when going from H_2O to D_2O. However, we have been able to determine (from spectra of **12** not only in H_2O and D_2O but also in an equimolar mixture of the two) that the Δ values of peaks 15 and 16 of Figure 2C are 0.12 ± 0.01 ppm. On this basis, we assign these peaks to M_B2(**12a**) and M_C2(**12**) but not on a one-

(21) Allerhand, A.; Doddrell, D.; Komoroski, R. *J. Chem. Phys.* **1971**, *55*, 189–198.

to-one basis. One-to-one assignments for these two resonances, *independent* of the above nonspecific assignments based on Δ values, can be made from consideration of the chemical shifts of C-2 of **1a**, **11a**, and **13**, as follows. First, the chemical shift of C-2 of **1a** (72.15 ppm in Table I) remains unchanged upon α-D-mannosylation of C-6 (72.18 ppm for M_A of **11a** in Table IV). If this is the case for a reducing α-D-mannopyranose, it is reasonable to assume that α-D-mannosylation of C-6 of a nonreducing α-D-mannopyranose will also have a negligible effect on the chemical shift of C-2. On this basis, the chemical shift of M_C2(**12a**) should be identical with that of C-2 of **13**. The latter is 71.41 ppm (Table IV), a value essentially identical with that of peak 15 (71.42 ppm) and significantly different from that of peak 16 (71.32 ppm). On this basis, we assign peak 15 to M_C2(**12a**). Furthermore, if α-D-mannosylation at C-6 does not affect the chemical shift of C-2, then the chemical shift of M_B2(**12a**) should be identical with that of M_B2(**11a**). The latter is 71.35 ppm, which indicates that M_B2(**12a**) should be assigned to peak 15 (71.42 ppm) or peak 16 (71.32 ppm) and *suggests* that peak 16 is the more likely assignment. Additional evidence for the *relative* assignments of M_B2 and M_C2 of **12a** comes from the peak heights of peaks 15 and 16 (Figure 4C). The smaller peak height of peak 16 is consistent with an assignment to M_B2 instead of M_C2, as a result of a slight chemical shift nonequivalence between M_B2(**12a**) and M_B2(**12b**), while M_C2(**12a**) and M_C2(**12b**) should have identical chemical shifts.

By elimination, peak 10 (72.23 ppm), peak 11 (72.19 ppm), one-half of peak 12 (72.04 ppm), and peak 13 (71.97 ppm) must arise from C-3 of all three α-D-mannopyranose residues and from M_A2(**12a**). We assign peak 13 (71.97 ppm) to M_A3(**12a**) because the chemical shift of M_A3(**11a**) is 71.91 ppm (Table IV). Each of the following three independent arguments is enough to assign one-half of peak 12 to M_C3(**12a**): (i) M_B3(**11a**), at 72.01 ppm in Table IV, should be an excellent model for M_C3(**12a**), because the only difference between M_B(**11a**) and M_C(**12a**) is that C-1 of M_B(**11a**) is linked to a reducing α-D-mannopyranose while C-1 of M_C(**12a**) is linked to a nonreducing α-D-mannopyranose; it is very unlikely that this difference can be transmitted to the chemical shift of C-3. (ii) The chemical shift of C-3 of **13** is 72.29 ppm (Table IV), which strongly suggests that the chemical shift of M_C3(**12a**) should be about 72.08 ppm, because α-D-mannosylation at C-6 of α-D-mannopyranose causes a 0.21 ppm *downfield* shift of the resonance of C-3 (compare the chemical shift of C-3 of **1a** in Table I with that of M_A3 of **11a** in Table IV). (iii) The chemical shift of peak 12 (72.04 ppm) is inconsistent with an assignment to M_A2(**12a**) because M_A3(**12a**) is only 0.07 ppm upfield of peak 12 (peak 13 at 71.97 ppm), and our chemical shifts for M_A2(**11a**) and M_A3(**11a**) are 72.13 and 71.91 ppm, respectively, with a separation of 0.22 ppm (Table IV); also, the chemical shift of peak 12 is inconsistent with an assignment to M_B3(**12a**), for reasons presented below.

By elimination, peaks 10 and 11 of Figure 4C must arise from M_A2(**12a**) and M_B3(**12a**). Each of the following two arguments provides independent confirmation that M_B2(**12a**) gives rise either to peak 11 (72.19 ppm) or peak 10 (72.23 ppm), most likely the latter: (i) We expect M_B3(**12a**) at about 72.23 ppm, because M_B3(**11a**) is at 72.01 ppm (Table IV) and, as explained above, α-D-mannosylation at C-3 of an α-D-mannopyranose residue should cause a downfield shift of about 0.21 ppm. (ii) C-3 of **13**, at 72.29 ppm (Table IV), is a good model for M_B3(**12a**), because the structural differences between M_B(**12a**) and the repeating unit of **13** are too far to cause significant chemical shift differences between M_B3(**12a**) and C-3 of **13**.

The above evidence *suggests* that M_B3(**12a**) should be assigned to peak 10 and not peak 11. This marginal specific assignment is confirmed by means of an independent marginal preference for the specific assignment of M_A2(**12a**) to peak 11 (72.19 ppm) over peak 10 (72.23 ppm), because our chemical shift of M_A2(**11a**) is 72.13 ppm (Table IV).

In the region of the C-4 resonances (peaks 17–20 of Figure 4C), peaks 18 and 20 are assigned to M_A4 of **12a** and **12b**, respectively, on the basis of their intensities and also on the basis of the similarity of their chemical shifts to those of the corresponding resonances of **11** (68.14 and 67.93 ppm in the spectra of both compounds). The one-to-one assignments of peak 17 (68.21 ppm) and peak 19 (68.08 ppm) to M_C4 and M_B4, respectively, of both anomers of **12** can be made on the basis of the following two arguments, each of which is sufficient evidence. (i) The chemical shift of M_B4(**11a**), at 68.18 ppm in Table IV, should be nearly identical with that of M_C4(**12**). (ii) The chemical shift of C-4 of **13**, at 68.04 ppm in Table IV, should be nearly identical with that of M_B4(**12a**).

In the region of glycosidically linked C-6 (peaks 21–23 of Figure 4C), relative intensities indicate that peak 21 (67.11 ppm), peak 22 (67.04 ppm), and peak 23 (66.95 ppm) should be assigned to M_A6(**12b**), M_A6(**12a**), and M_B6 of both anomeric species, respectively. The relative assignments of M_A6 and M_B6 of **12a** are confirmed by noting that they yield a chemical shift for M_A6(**12a**) which is much closer to the value of 67.17 ppm observed for M_A6(**11a**) (Table IV) than would be the case if the assignments were reversed. Also, our chemical shift for M_B6(**12a**) is much closer to the value for C-6 of **13** (66.83 ppm in Table IV) than if the assignments were reversed.

In the region of unlinked C-6 resonances, there is only one resonance in the spectrum of **12** (peak 24 at 62.37 ppm in Figure 4C) which must arise from M_C6.

This completes our assignments for all the compounds examined in this study. It is now possible to draw some conclusions about the effects of α-D-mannosylation at C-2, C-3, C-4, and C-6 of α-D-mannopyranose residues on the chemical shifts of all the carbons of the residue which has been mannosylated.

Conclusions

In Table V we summarize the effects of α-D-mannosylation (at C-2, C-3, C-4, and C-6 of an α-D-mannopyranosyl or β-D-mannopyranosyl residue) on the chemical shift of all the carbons of the mannosylated residue. In each case, these results were obtained by taking the chemical shifts of a residue located second from the nonreducing end of the oligosaccharide and subtracting a "reference" chemical shift of the corresponding carbon of the nonreducing terminal residue of the oligosaccharide which results from the removal of the nonreducing residue of the original oligosaccharide. For example, the effects of substitution at C-2 of free α-D-mannopyranose (**1a**) were obtained by subtracting the chemical shifts of **1a** from the corresponding ones of the reducing residue (M_A) of Manα1→2Man(α) (**6a**), and the effects of substitution at C-2 of the nonreducing terminal residue (M_B) of **6a** were determined by subtracting the chemical shifts of M_B of **6a** from the corresponding ones of the central residue (M_B) of Manα1→2Manα1→2Man(α) (**7a**).

Our results in Table V can be compared with the corresponding ones for the effects of *O*-methyl substitution reported by Gorin,[14] except for 6-*O*-substitution of α-D-mannopyranose, which was not reported by Gorin. For the most part, the *directions* of the chemical shift displacements upon methylation are the same as for α-D-mannosylation (C-1 of β-mannose upon substitution at C-2 and C-3 of α-mannose upon substitution at C-4 are notable exceptions). However, there is a significant difference in the *magnitude* of many displacements. Clearly *O*-methylation effects must be used with great caution in the interpretation of ^{13}C NMR spectra of oligosaccharides.

In the following paper in this issue,[6] we use the effects of α-D-mannosylation (Tables V and VI) to interpret the ^{13}C NMR spectra of compounds of the type (Manα)$_n$Manβ1→4GlcNAcβ1→4GlcNAcβ1→Asn, which occur commonly as side chains of glycoproteins.[9] Table V indicates that α-D-mannosylation at C-2 of an α-D-mannopyranose residue causes significant chemical shift changes for all carbons except C-5 and C-6. In the case of a β-D-mannopyranose residue, α-D-mannosylation at C-2 significantly affects all carbons except C-4 and C-6. The α-D-mannosylation of C-3 of an α-D-mannopyranose affects only C-2, C-3, and C-4, while α-D-mannosylation of C-4 has large effects on the chemical shifts of C-2, C-3, C-4, and C-5. The α-D-mannosylation of C-6 of an α- or β-D-mannopyranose residue

Table V. Changes in the ^{13}C Chemical Shifts of α-D-Mannopyranosyl and β-D-Mannopyranosyl Residues Upon α-D-Mannosylation of one of the Nonanomeric Carbons

type linkage[a]	modified residue[b]	intact residue[c]	chemical shifts change[d] C-1	C-2	C-3	C-4	C-5	C-6
$2\alpha_R$	M_A(6a)	**1a**	−1.53	+8.23	−0.31	+0.17	+0.02	+0.04[e]
2α	M_B(7a)	M_B(6a)	−1.63	+8.34	−0.37[e]	+0.22[e]	+0.01	*f*
2α	M_C(**8a**)	M_C(7a)	−1.68[e]	+8.58[e]	−0.45	+0.18	−0.01	*g*
$2\beta_R$	M_A(6b)	1b	−0.27	+6.88	+0.32	+0.09[e]	+0.52	−0.17
3α	M_C(9a)	M_C(7a)	−0.08[e]	−0.42[e]	+7.45	−0.70	+0.05[e]	*h*
3β	*i*	*j*	0	−0.4	+7.8	−0.6	−0.1	+0.1
4α	M_A(**10**)	2	−0.14	+0.49	+0.42	+7.36[k]	−1.50	+0.10[l]
$6\alpha_R$	M_A(**11a**)	1a	+0.14	−0.02	+0.21	−0.16	−1.79	+4.75
6α	M_B(**12a**)	M_B(**11a**)	−0.02	−0.03	+0.22	−0.10	−2.00[m]	+4.59
6α	13	M_C(**12a**)	+0.02	−0.01	+0.25	−0.17	−2.05	+4.46
$6\beta_R$	M_A(**11b**)	**1b**	+0.16	−0.06	+0.12	−0.13	−1.88	+4.80

[a] The number indicates the carbon (of the mannose residue under consideration) which is linked to C-1 of an α-D-mannopyranosyl residue. The greek letter indicates if the residue under consideration is in the α-pyranose or β-pyranose configuration. The subscript R (when present) indicates that the residue under consideration is a reducing one. [b] Each entry in this column lists a suitably α-D-mannosylated α-D-mannopyranose or β-D-mannopyranose residue whose ^{13}C chemical shifts were taken as the ones "modified upon α-D-mannosylation". [c] Each entry in this column is a terminal α-D-mannopyranosyl or β-D-mannopyranosyl residue which results from the residue listed in the "modified residue" column upon removal of the α-D-mannopyranosyl residue linked to the pertinent nonanomeric carbon. [d] Chemical shift of the "modified residue" *minus* the one of the "intact residue" (taken from Figure 1A and Tables II and IV). [e] Because of a lack of one-to-one assignments, this is one of two possible values. The other value differs by less than 0.1 ppm from the listed one. [f] Because of a lack of one-to-one assignment several values in the range −0.04 to +0.11 ppm are possible (see Table II). [g] Because of a lack of one-to-one assignments, several values in the range −0.11 to +0.04 ppm are possible (see Table II). [h] Because of a lack of one-to-one assignments, several values in the range −0.04 to +0.08 ppm are possible (see Table II). [i] β-D-mannopyranosyl residue of Manα1→3Manβ1→4GlcNAc in D_2O, taken from Table I of Nunez, H. A.; Matsuura, F.; Sweeley, C. C. *Arch. Biochem. Biophys.* **1981**, *212*, 638–643. We subtracted 0.1 ppm from each reported chemical shift, in order to improve the consistency with our referencing system, on the basis of our chemical shifts of glycopeptides.[6] [j] β-D-mannopyranosyl residue of Manβ1→4GlcNAcβ1→4GlcNAcβ1→Asn in D_2O. The chemical shifts are presented in the following paper in this issue.[6] [k] Because of a lack of one-to-one assignments, another possible value is 6.84 ppm (see Table IV). [l] Because of a lack of one-to-one assignments, another possible value is −0.02 ppm (see Table IV). [m] Because of a lack of one-to-one assignments, another possible value is −2.15 ppm (see Table IV).

Table VI. Effect of Mannosylation at the Anomeric Carbon on the ^{13}C Chemical Shifts of α-D-Mannopyranose: Chemical Shifts of Nonreducing Terminal α-D-Mannopyranose Residues Involved in 1→2, 1→3, 1→4, and 1→6 Linkages to Other Mannose Residues

link[a]	compd[b]	chemical shift and effect of mannosylation, ppm[c] C-1	C-2	C-3	C-4	C-5	C-6
$1\rightarrow2\alpha_R$	6a	103.44	71.39	71.77	68.27	74.54	62.36[d]
		(+8.00)	(−0.76)	(+0.07)	(−0.03)	(+0.79)	
$1\rightarrow2\alpha$	7a	103.47	71.40[d]	71.83	68.27	74.55	62.41[d]
		(+8.03)	(−0.75)	(+0.13)	(−0.03)	(+0.80)	
$1\rightarrow2\beta_R$	6b	102.70	71.52	71.68	68.06[d]	74.18	*e*
		(+7.26)	(−0.63)	(−0.02)	(−0.24)	(+0.43)	
$1\rightarrow3\alpha$	9a	103.46[d]	71.46[d]	71.82	68.31	74.67[d]	62.33[f]
		(+7.95)	(−0.69)	(+0.12)	(+0.01)	(+0.92)	
$1\rightarrow3\beta$	*g*	103.7	71.8	72.0	68.4	74.8	62.6
		(+8.3)	(−0.4)	(+0.3)	(+0.1)	(+1.1)	(+0.2)
$1\rightarrow4\alpha$	**10**	102.74	71.69	71.81	68.00	75.03[h]	62.34[i]
		(+7.30)	(−0.46)	(+0.11)	(−0.30)	(+1.28)	(−0.07)
$1\rightarrow6\alpha_R$	11a	100.86	71.35	72.01	68.18	74.04	62.36
		(+5.42)	(−0.80)	(+0.31)	(−0.12)	(+0.29)	(−0.05)
$1\rightarrow6\alpha$	**12a**	100.63	71.42	72.04	68.21	74.07	62.37
		(+5.19)	(−0.83)	(+0.34)	(−0.09)	(+0.32)	(−0.04)
$1\rightarrow6\beta_R$	**11b**	100.88	71.3	72.0	68.2	74.00	62.4
		(+5.45)	(−0.9)	(+0.3)	(−0.1)	(+0.25)	(0)

[a] The designations $1\rightarrow2\alpha_R$ and $1\rightarrow2\alpha$ indicate that the nonreducing terminal α-D-mannopyranose under consideration is involved in a 1→2 linkage to a *reducing* and *nonreducing* α-D-mannopyranose, respectively. [b] Specific compound whose chemical shifts (of the nonreducing terminal α-D-mannopyranose residue) have been tabulated. [c] Chemical shifts are in ppm downfield from Me_4Si (taken from Tables II and IV). Each effect of mannosylation (number in parentheses under each chemical shift) was obtained by subtracting the chemical shift of the corresponding carbon of free α-D-mannopyranose (from Table I) from the listed chemical shift. [d] Because of a lack of one-to-one assignments, this is one of two possible values. The other value differs by less than 0.1 ppm from the listed one (see Table II). [e] Not detected. Probably identical with the corresponding value of 6a. [f] Another possibility is 62.49 ppm (see Table II). [g] The listed chemical shifts are those reported for the α-D-mannopyranose residue of Manα1→3Manβ1→4GlcNAc in Table I of Nunez, H. A.; Matsuura, F.; Sweeley, C. C. *Arch. Biochem. Biophys.* 1981, *212*, 638–643. We subtracted 0.1 ppm from each of the reported values in order to improve the consistency with our chemical shift referencing system, on the basis of our chemical shifts of glycopeptides.[6] Furthermore, we then *added* 0.2 ppm to the reported chemical shift of C-3 and 0.1 ppm to those of C-2, C-4, and C-6, in order to correct for the fact that the reported values were obtained for a sample in D_2O, while our chemical shifts correspond to compounds in H_2O (see text and Table III). [h] A second possible value is 75.55 ppm (see Table IV). [i] A second possible value is 62.45 ppm (see Table IV).

has large effects on C-5 and C-6 and small but significant effects on some other carbons (C-3 and C-4 of both anomers and C-1 of the β anomer).

It follows from the above that the chemical shifts of most carbons of a mannose residue are affected in specific and differing ways by mannosylation at C-2, C-3, C-4, and C-6. This phenomenon enhances the likelihood of observing many resolved single-carbon resonances in ^{13}C NMR spectra of mannose-containing glycopeptides.

The likelihood of highly resolved ^{13}C NMR spectra for com-

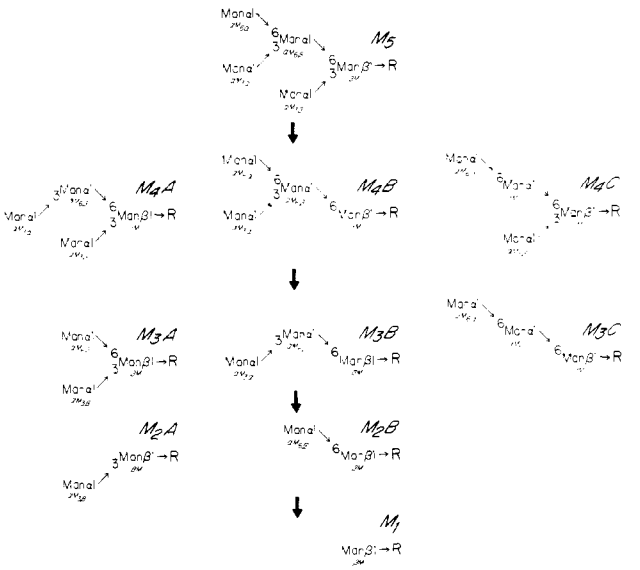

Figure 5. Possible degradation products of $(Man\alpha)_4Man\beta1\rightarrow$ $4GlcNAc\beta1\rightarrow4GlcNAc\rightarrow Asn$ (structure M_5) upon treatment with jack bean α-mannosidase. The pathway indicated with thick arrows is the one that occurs experimentally.[5] R = $4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow Asn$.

pounds of the type $(Man\alpha)_nMan\beta1\rightarrow4GlcNAc\beta1\rightarrow$ $4GlcNAc\beta1\rightarrow Asn$ is also enhanced by another phenomenon: The ^{13}C chemical shifts of *terminal* α-D-mannopyranosyl residues are influenced by the nature of the glycosidic linkage to other mannose residues. In Table VI we summarize the chemical shifts of the nonreducing terminal mannose residues presented in this report, together with chemical shift differences relative to the corresponding free monosaccharide. It can be seen that the chemical shift of C-1 of a terminal α-D-mannopyranose occurs at about 103.4 ppm if it is linked to C-2 or C-3 of another α-D-mannopyranose (or C-3 of a β-D-mannopyranose[11]), at about 102.7 ppm if it is linked to C-2 of a β-D-mannopyranose or C-4 of an α-D-mannopyranose, at about 100.9 ppm if it is linked to C-6 of a reducing α- or β-D-mannopyranose, and at about 100.6 ppm if it is linked to C-6 of a nonreducing α-D-mannopyranose residue. These results are in agreement with analogous ones reported by Gorin.[11] As will be seen in the following paper in this issue, they greatly facilitate the analysis of spectra of glycopeptides.

However, not only the anomeric carbons of nonreducing terminal α-D-mannopyranose residues have chemical shifts which are influenced by the type of linkage at C-1. Table VI indicates that C-2, C-3, C-4, and C-5 are also affected. In the case of C-2, a $1\rightarrow6$ linkage yields a chemical shift of about 71.3 ppm, a $1\rightarrow2$ or $1\rightarrow3(\alpha)$ linkage yields 71.4–71.5 ppm, and a $1\rightarrow4(\alpha)$ yields about 71.7 ppm. In the case of C-3, we get about 72.0 ppm for a $1\rightarrow6$ linkage and about 71.7–71.8 ppm for the other linkages. In the case of C-4, we have about 68.0 ppm for $1\rightarrow4(\alpha)$ and $1\rightarrow2(\beta)$ and 68.2–68.3 ppm for the other linkages. For C-5 we observe about 74.0 ppm for $1\rightarrow6$, about 74.2 ppm for $1\rightarrow2(\beta)$, about 74.5–74.7 ppm for $1\rightarrow2(\alpha)$ and $1\rightarrow3(\alpha)$, and about 75.0 or 75.5 ppm for a $1\rightarrow4(\alpha)$ linkage. Although most of these differences are small, many of them are large enough to yield well-resolved single-carbon resonances for the various terminal α-D-mannopyranosyl residues of high mannose glycopeptides. These effects are also useful in the interpretation of the ^{13}C NMR spectra of mannose-containing glycopeptides, as shown in the following paper.

We now propose that the data of Tables V and VI should suffice for making fairly accurate predictions of the ^{13}C NMR spectra of linear or branched chains of α-D-mannopyranosyl residues, as follows. The starting point for each residue is one set of chemical shifts taken from Table VI, chosen on the basis of the type of linkage present at C-1 of the residue under consideration. For example, if C-1 is linked to C-2 of a β-D-mannopyranosyl residue, then the second set of chemical shifts listed in Table VI (for the $1\rightarrow2\beta_R$ link) is the correct choice. Next, the chosen set of chemical

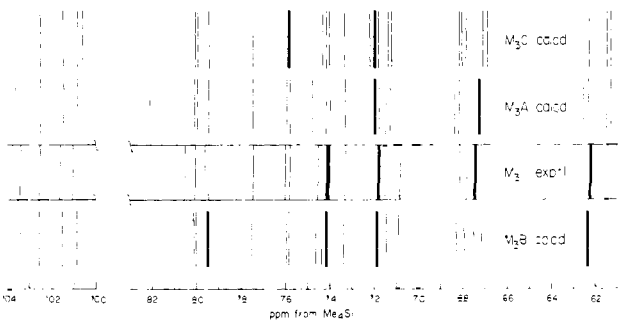

Figure 6. A comparison of the calculated ^{13}C NMR spectra of M_3A, M_3B, and M_3C (structures of Figure 5), obtained as described in the text, with the experimental spectrum of a compound of the type $(Man\alpha)_2Man\beta1\rightarrow4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow Asn$ (designated M_3), taken from ref 5. Thin and thick lines designate single-carbon and two-carbon resonances, respectively. The very thick line at about 74 ppm in the experimental spectrum is a three-carbon resonance. The resonances of the asparagine and those of C-2 and the acetyl groups of the GlcNAc residues are not in the spectral range shown.

Table VII. Comparison of the Experimental Spectrum of $(Man\alpha1\rightarrow)_3Man\beta1\rightarrow4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow Asn$ with Predicted Spectra for Structures M_3A, M_3B, and M_3C of Figure 5

spectral region, ppm[a]	number of carbons[b]			
	exptl	M_{3B}	M_{3A}	M_{3C}
103.5–103.7	1	1	1	0
100.5–102.0	2	2	2	3
~82	0	0	1	0
79.0–80.5	4	4	3	3
74.0–75.0	4	4	3	3
71.3–72.3	3	3	5	6
~70.8	1	1	0	0
67.0–68.5	4	4	4	5
62.2–62.6	2	2	2	1
discrepancies[c]		0	5	8

[a] We are considering the range of chemical shifts 61–104 ppm (Figure 6). Some carbons have chemical shifts outside the regions listed in this table, but they have been omitted because their chemical shifts are essentially the same in all the spectra of Figure 6. [b] Taken from Figure 6. [c] Total number of spectral regions in which there is a discrepancy of one or more carbons between the experimental and calculated spectrum.

shifts from Table VI is modified on the basis of Table V to take into account any linkages (of α-D-mannopyranosyl residues) at C-2, C-3, C-4, or C-6 of the residue under consideration. We shall now present an application of this method which will illustrate the good agreement between observed spectra and those predicted on the basis of Tables V and VI.

In a previous publication,[5] we reported on the kinetics of jack bean α-mannosidase action on various α-D-mannopyranosyl linkages in two glycopeptides, one of which is shown as structure M_5 in Figure 5, where R = $4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow Asn$. All the possible structures which can result from the successive hydrolysis of terminal α-D-mannopyranose residues are also shown in Figure 5. Before our report, there was a belief that jack bean α-mannosidase hydrolyzes $1\rightarrow6$ linkages at least 15 times faster than $1\rightarrow3$ linkages. On this basis, the removal of one mannose from M_5 would yield mainly M_4A, which would then yield a mixture of mainly M_3B and M_2A; note that M_3A, when formed, would rapidly lose the $(1\rightarrow6)$-linked residue $\alpha M_{6\beta}$ (see Figure 5). We were able to monitor the actual progress of the hydrolysis by carrying out the reaction in an NMR tube,[5] and we were able to show with the use of a few chemical shifts of model compounds that M_4B and M_3B are the main intermediates in the hydrolysis, which implies that the $1\rightarrow3$ linkage of $\alpha M_{3\beta}$ is hydrolyzed much faster than the $1\rightarrow6$ linkage of $\alpha M_{6\alpha}$ (Figure 5). With the use of Tables V and VI, we no longer have to rely on a few chemical shifts of model compounds to determine the structures of the intermediates. In Figure 6 we show the observed spectrum[5] of the intermediate with three mannose residues (designated "M_3

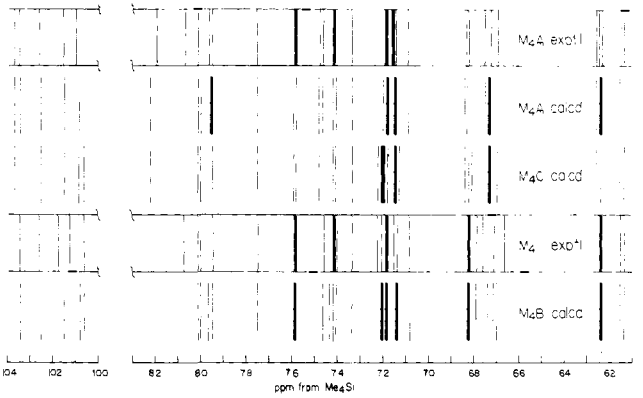

Figure 7. A comparison of the calculated ^{13}C NMR spectra of M_4A, M_4B, and M_4C (structures of Figure 5), obtained as described in the text, with the experimental ones of M_4A, taken from ref 6, and a compound of the type $(Man\alpha)_3Man\beta1\rightarrow4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow Asn$ (designated M_4), taken from ref 5. Thin and thick lines designate single-carbon and two-carbon resonances, respectively. The very thick line at about 72 ppm is the calculated spectrum of M_4C is a three-carbon resonance. The resonances of the asparagine and those of C-2 and the acetyl groups of the GlcNAc residues are not in the spectral range shown.

Table VIII. Comparison of the Experimental Spectrum of $(Man\alpha1\rightarrow)_3Man\beta1\rightarrow4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow Asn$ with Predicted Spectra for Structures M_4A, M_4B, and M_4C of Figure 5

spectral region, ppm[a]	number of carbons[b]			
	exptl	M_4B	M_4A	M_4C
103.5-103.7	1	1	2	1
100.5-102.0	3	3	2	3
~82	0	0	1	1
79.0-81.0	4	4	4	3
74.0-75.0	4	4	4	3
71.3-72.3	6	6	5	8
70.8-70.9	1	1	1	0
66.5-68.5	6	6	5	6
62.2-62.6	2	2	3	2
discrepancies[c]		0	6	5

[a] We are considering the range of chemical shifts 61-104 ppm (Figure 7). Some carbons have chemical shifts outside the regions listed in this table, but they have been omitted because their chemical shifts are essentially the same in all the spectra of Figure 7. [b] Taken from Figure 7, except that the experimental spectrum of M_4A (which in each region shows the same number of carbons as the calculated spectrum of M_4A) is not present in the table. [c] See footnote *c* of Table VII.

exptl") and the calculated spectra of M_3A, M_3B, and M_3C.[22] In Table VII we show the number of resonances in some spectral regions of interest for the experimental spectrum and for the calculated ones. Clearly, it follows from Figure 6 and even more dramatically from Table VII that only the structure M_3B (Figure 5) is compatible with the observed spectrum of the compound with three remaining mannose residues. Furthermore, the experimental spectrum after removal of only one mannose from M_5, shown as "M_4 exptl" in Figure 7, is only compatible with structure M_4B (Figure 7 and Table VIII). As a final test of the use of Tables V and VI, the compound M_4A, prepared as described in the following paper of this issue,[6] yielded the spectrum labeled "M_4A exptl" in Figure 7, which is in good agreement with the calculated one for M_4A.

Although there is good agreement between our experimental and calculated spectra for M_3B, M_4B, and M_4A, it is not perfect (Figures 6 and 7). The small discrepancies are best discussed after specific assignments for all the resonances in the observed spectra of these compounds are presented in the following paper of this issue. It is noteworthy that such specific assignments are not needed in order to use Tables V and VI to make the structural determinations presented in Figures 6 and 7.

In summary, there is a great diversity of chemical shift variations in the ^{13}C NMR spectra of mannose oligosaccharide chains, which should contribute to resolution (and complexity) of ^{13}C NMR spectra of glycopeptides and carbohydrate side chains of glycoproteins. We believe that the results presented here should facilitate the increased use of ^{13}C NMR for studies of mannose-containing glycopeptides and glycoproteins. Clearly, additional studies of more complex model oligosaccharides, with other types of residues besides mannose, will be necessary in order to set up a full library of ^{13}C chemical shifts and chemical shift variations pertinent to studies of glycopeptides and glycoproteins.

It is obviously desirable to extend the methodology presented in this report to a much greater variety of oligosaccharide systems than the ones we have studied here. Clearly, this methodology requires rigorous assignments of resonances and precise and consistent chemical shift data. With respect to chemical shifts, the literature is plagued by inconsistencies in ^{13}C chemical shifts of carbohydrates, even monosaccharides. As an illustration, consider the 12 sets of reported chemical shifts of the β-pyranose anomer of D-mannose[12,13c,d,14,15,17,20,23] (not a complete list) shown in Table IX. Data sets 1-11 of Table IX are all chemical shifts relative to Me_4Si. Nevertheless, there is a difference of over 2 ppm between sets 1 and 11. It follows from Table III that the use of H_2O in some cases and D_2O in others can cause discrepancies no greater than about 0.15 ppm. It follows from a comparison of data sets 7 and 8 of Table IX (recorded in the same laboratory on the same instrument and with identical referencing procedures) that when going from dilute (0.09 M) to very concentrated (4.0 M) solutions, the chemical shift changes are $\lesssim$0.1 ppm. It is possible (but subject to verification) that the increase in chemical shifts when going from sets 5-8 to sets 9 and 10 in Table IX is caused by the increase in sample temperature. However, the large difference between set 11 (60 °C) and sets 9 (70 °C) and 10 (50 °C) are obviously not caused by temperature changes. The lion's share of the discrepancy between any two data sets of Table IX can be removed by adding a constant factor to one of the sets, which is evidence of differences in the method of choosing the ^{13}C chemical shift of Me_4Si. We present in Table X a revised listing of data sets 1-12 of Table IX, obtained as follows. A constant factor was added to each data set of Table IX, such that the chemical shift of C-1 would become 95.06 ppm (samples in H_2O) or 94.96 ppm (samples in D_2O). Table X shows the discrepancies between the 12 data sets, independent of referencing differences. Clearly, significant discrepancies remain. For samples in D_2O at about 35 °C, the "normalized" chemical shift of C-4 ranges from 62.14 to 62.46 ppm (Table X).

Another example which illustrates the need for improved precision of reported ^{13}C chemical shifts of carbohydrates is given in Table XI, which shows two reported sets of ^{13}C chemical shifts for the β-pyranose anomer of GlcNAc and the reducing residue of $GlcNAc\beta1\rightarrow4GlcNAc(\beta)$.[24,25] When we take the chemical shifts of the reducing residue of $GlcNAc\beta1\rightarrow4GlcNAc(\beta)$ and subtract the corresponding values of monomeric $GlcNAc(\beta)$, we get the effects of substitution at C-4 of $GlcNAc(\beta)$ by another $GlcNAc(\beta)$ residue (the δ values of Table XI). Even though the chemical shifts reported by Colson and King[24] may differ from those of Nunez and Barker[25] because of variations in the exact method of chemical shift referencing,[24,25] clearly the δ values should be independent of referencing. Nevertheless, there are differences of up to 0.3 ppm between the two sets of δ values (Table XI),

(22) Our calculated chemical shifts for the β-D-mannopyranosyl residue and for the two GlcNAc residues of the structures shown in Figure 5 (where R = $4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow$Asn) are based on the chemical shifts of $Man\beta1\rightarrow4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow$Asn presented in the following paper of this issue,[6] together with the effects of substitution at C-3 and C-6 of the β-D-mannopyranosyl residue which are shown in Table V.

(23) (a) Gorin, P. A. J. *Can. J. Chem.* **1973**, *51*, 2105-2109. (b) Friebolin, H.; Keilich, G.; Frank, N.; Dabrowski, U.; Siefert, E. *Org. Magn. Reson.* **1979**, *12*, 216-222.
(24) Colson, P.; King, R. R. *Carbohydr. Res.* **1976**, *47*, 1-13.
(25) Nunez, H. A.; Barker, R. *Biochemistry* **1980**, *19*, 489-495.

Table IX. Reported Carbon-13 Chemical Shifts for Aqueous β-D-Mannopyranose

	chemical shift[a]									
data set	C-1	C-2	C-3	C-4	C-5	C-6	solvent	concn,[b] M	temp, °C	ref
1	94.1	71.8	73.6	67.1	76.7	61.6	D_2O	*c*	27	23a
2	94.24	71.83	73.65	67.22	76.75	61.63	D_2O	~0.9	33	13c
3	94.25	71.80	73.67	67.22	76.65	61.67	D_2O	*d*	*d*	17
4	94.35	71.97	73.82	67.37	76.68	61.80	H_2O	*d*	*d*	17
5	94.6	72.3	74.1	67.8	77.2	62.1	D_2O	*d*	33	13d
6	94.96	72.52	74.36	67.93	77.45	62.29	D_2O	0.09	33	this work
7	95.05	72.66	74.51	68.11	77.37	62.51	H_2O	4.0	36	12
8	95.06	72.67	74.51	68.06	77.48	62.41	H_2O	0.09	33	this work
9	95.2	72.8	74.7	68.5	77.7	62.7	D_2O	~0.14	70	14
10	95.2	72.8	74.8	68.3	77.6	62.6	H_2O	*d*	50	15
11	96.5	74.0	75.8	69.5	79.0	63.8	D_2O	*e*	60	23b
12	96.7	74.2	76.0	69.6	79.1	63.9	D_2O	2.8	36	20

[a] All chemical shifts are reported in ppm downfield from Me_4Si, except those from ref 20, which are referenced relative to the methyl carbon resonance of sodium 3-(trimethylsilyl)-1-propanesulfonate (last entry in the table). We have arranged the reported chemical shifts of β-D-mannopyranose so that the value for C-1 increases monotonically from top to bottom. [b] Total D-mannose concentration. [c] In the range 0.5–1.5 M. [d] Not reported. [e] In the range 0.3–1.1 M.

Table X. Adjusted Carbon-13 Chemical Shifts of Aqueous β-D-Mannopyranose

	chemical shift[b]								
data set[a]	C-1	C-2	C-3	C-4	C-5	C-6	solvent	concn,[c] M	temp, °C
8	95.06	72.67	74.51	68.06	77.48	62.41	H_2O	0.09	33
4	95.06	72.68	74.53	68.08	77.39	62.51	H_2O	*d*	*d*
7	95.06	72.67	74.52	68.12	77.38	62.52	H_2O	4.0	36
10	95.06	72.66	74.66	68.16	77.46	62.46	H_2O	*d*	50
6	94.96	72.52	74.36	67.93	77.45	62.29	D_2O	0.09	33
1	94.96	72.66	74.46	67.96	77.56	62.46	D_2O	*e*	27
2	94.96	72.55	74.37	67.94	77.47	62.35	D_2O	~0.9	33
3	94.96	72.51	74.38	67.93	77.36	62.38	D_2O	*d*	*d*
5	94.96	72.66	74.46	68.16	77.56	62.46	D_2O	*d*	33
9	94.96	72.56	74.46	68.26	77.46	62.46	D_2O	~0.14	70
11	94.96	72.46	74.26	67.96	77.46	62.26	D_2O	*f*	60
12	94.96	72.46	74.26	67.86	77.36	62.14	D_2O	2.8	36

[a] Data set designations of Table IX. [b] The chemical shifts of data set 8 (in H_2O) and data set 6 (in D_2O) are taken directly from Table IX. For all the other data sets, the values of Table IX were modified by an additive constant that would make the chemical shift of C-1 equal to the value of either data set 8 (samples in H_2O) or data set 6 (samples in D_2O). [c] Total D-mannose concentration. [d] Not reported. [e] In the range 0.5–1.5 M. [f] In the range 0.3–1.1 M.

Table XI. A Comparison of Reported ^{13}C Chemical Shifts for GlcNAc(β) Residues[a]

	Colson and King[b]			Nunez and Barker[c]		
carbon	GlcNAcβ1→4 GlcNAc(β)[d]	GlcNAc(β)[e]	δ[f]	GlcNAcβ1→4 GlcNAc(β)[d]	GlcNAc(β)[e]	δ[f]
1	96.2	96.2	0	96.2	96.4	−0.2
2	57.4	58.0	−0.6	57.5	58.1	−0.6
3	73.8	75.2	−1.4	73.6	75.3	−1.7
4	80.7	71.2	+9.5	80.9	71.2	+9.7
5	75.8	77.2	−1.4	75.9	77.3	−1.4
6	61.3	62.0	−0.7	61.5	62.2	−0.7
CH_3	23.4	23.5	−0.1	23.5	23.5	0

[a] Chemical shifts are given in parts per million downfield from the ^{13}C resonance of Me_4Si. [b] Taken from ref 24. Spectra were recorded at 20 or 25 MHz (50–75 mg/mL in D_2O, about 30 °C). Reported precision of the chemical shifts is ±0.1 ppm. [c] Taken from ref 25. Spectra were recorded at 15.1 or 90.5 MHz (0.05–0.2 M concentrations in D_2O). Reported precision of the chemical shifts is ±0.1 ppm. [d] Chemical shifts of the reducing β-pyranose residue. [e] Chemical shifts of the β-pyranose anomeric form of the monosaccharide. [f] Effect of substitution at C-4 of GlcNAc(β) by another GlcNAc(β) residue, obtained by subtracting each chemical shift of the monosaccharide from the corresponding value of the reducing residue of the disaccharide.

which probably result from the reported ±0.1 ppm precision of the chemical shifts.

On the basis of the above evidence, we feel that our data for substitution at C-3 of β-D-mannopyranose (entry 3β of Table V) and for the chemical shifts of a terminal α-D-mannopyranose linked to C-3 of a β-D-mannopyranose residue (entry 1→3β of Table VI) may yield less accurate predictions than the rest of Tables V and VI, because our parameters for 3β and 1→3β are based on chemical shifts measured in other laboratories and not our own (see footnotes i of Table V and g of Table VI).

With the increasing availability of high-field NMR instruments equipped with large computer memories (64K and larger data memories) it has become practical to measure ^{13}C chemical shifts with a digital resolution of 0.01 ppm or better. Therefore, it is realistic to expect that a large library of very precise ^{13}C chemical shifts of carbohydrates will develop rapidly and that the full ^{13}C NMR spectra of complex carbohydrates (and not just the anomeric carbon resonances) will be used in structural studies of these carbohydrates. In the following paper of this issue[6] we show that it is feasible to assign most of the ^{13}C resonances of large and complex oligosaccharides to specific carbons, even when the resonances are very closely crowded together, provided that the chemical shifts are measured with a precision much greater than ±0.1 ppm.

Experimental Section

Materials. D-mannose and methyl α-D-mannopyranoside were obtained from Sigma Chemical Co., St. Louis, MO. Samples of **6**, **8**, **9**,

10, 12, and 13 were kindly supplied by Professor Clinton E. Ballou, Department of Biochemistry, University of California, Berkeley. Samples of 7, 11, and 13 were kindly provided by Dr. Philip A. J. Gorin of the Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada. Compound 3 was prepared as described below. The ^{13}C chemical shifts of 4 and 5 wre taken directly from the report of Allerhand et al.[16]

Preparation of Ethyl α-D-Mannopyranoside. Gaseous HCl (about 5 g) was bubbled into a solution of 5 g of D-mannose in 100 mL of anhydrous ethanol. Then the solution was refluxed for about 1 h, during which its color darkened. After cooling to room temperature, silver carbonate was added until it no longer caused effervescence. The resulting mixture was filtered and lyophilized. The residue was dissolved in 100 mL of H_2O, treated with 5 g of charcoal, filtrered, and freeze dried.

Purification of Manα1→6Man. An impure sample of 11 was subjected to gel filtration chromatography on Biogel P2, 200–400 mesh (1.7 × 50 cm column), using H_2O as eluant (6 mL/h). The elution profile was monitored at 206 nm and at 540 nm (after orcinol/acid test). The fraction of lowest molecular weight which had a positive orcinol/acid test was collected and lyophilized.

Carbon-13 NMR Spectroscopy. The ^{13}C NMR spectra were recorded at 67.9 MHz, under conditions of proton decoupling, on a Fourier transform NMR spectrometer equipped with a Bruker 63.4-kG high-resolution superconducting magnet, Brucker 10- and 15-mm probes, "home-built" radiofrequency electronics (with quadrature detection), and a Nicolet 1085 computer. The probe temperature was about 32 °C. Unless otherwise indicated (see below), time-domain spectra were accumulated in 8192 addresses for each of the two detection channels, with a total spectral width of 6250 Hz (which corresponds to a digital resoltuion of 0.0112 ppm), and a recycle time of 1.32 s. The spectral width was 5000 Hz for Figure 4C, and it was 12500 Hz for Figures 2A,D and 4A. However, a spectral width of 6250 Hz was used for all the spectra of samples in H_2O and D_2O which were used to get the Δ values of Table III. Furthermore, except in the case of 9, all spectra used for getting Δ values were processed with 0.0056 ppm digital resolution, by adding a tail of 8192 zero addresses to each block of 8192 time-domain data points. In the cases of 11 and 12, which yielded several very closely spaced peaks, the one-to-one connections between resonances of samples in H_2O and in D_2O were established by also using mixtures of H_2O and D_2O as the solvent. Most spectra were recorded on solutions which contained a trace of dioxane as an internal reference (taken at 67.86 ppm downfield from the ^{13}C resonance of Me_4Si). However, in some cases, especially when the oligosaccharide had a resonance close to that of dioxane, spectra were recorded for samples with and without dioxane. Chemical shifts of spectra without dioxane (such as Figures 2C,D, 3, and 4A,C) were obtained by using a secondary reference after the chemical shifts of the sample with dioxane had been determined. All chemical shifts were measured digitally.

Acknowledgment. This research was supported by the National Science Foundation (Grant PCM 79-16899). We thank Professor Clinton E. Ballou and Dr. Philip A. J. Gorin for supplying the oligosaccharides used in this study. We thank Dr. Kilian Dill for carrying out the preparation of 3.

Systematic Approach to the Analysis of Carbon-13 NMR Spectra of Complex Carbohydrates. 2. Application to the High Mannose Glycopeptides of Hen Ovalbumin

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Contribution from the Department of Chemistry, Indiana University, Bloomington, Indiana 47405. *Received October 12, 1982*

Abstract: Seven glycopeptides of the type $(Man\alpha)_nMan\beta1\rightarrow4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow Asn$, where $n = 0, 2, 3, 4, 5$, and 6, are studied by ^{13}C NMR spectroscopy at 67.9 MHz. Even the spectrum of the compound with $n = 6$ yields well-resolved single-carbon resonances for most carbons. Nearly all the resonances of all seven compounds are assigned to specific carbons with the use of the following methods: (i) the empirical parameters based on small oligosaccharides presented in the preceding paper of this issue; (ii) comparisons of spectra within the series of glycopeptides from $n = 0$ to 6; (iii) comparisons of chemical shifts of compounds in H_2O with those of the same compounds in D_2O; (iv) the effect of pH on chemical shifts; (v) spin–lattice relaxation times; and (vi) values of directly bonded ^{13}C–^1H scalar coupling constants. It is shown that the ^{13}C NMR spectrum of a glycopeptide with n α-D-mannopyranose residues can be predicted with great accuracy from a knowledge of the spectrum of any related glycopeptide with $n - 1$ residues, with the use of the parameters presented in the preceding paper of this issue.

In the preceding paper of this issue[1] we presented chemical shifts and assignments for the ^{13}C resonances of simple mannose oligosaccharides. Here we present the interpretation of the ^{13}C NMR spectra of the high mannose glycopeptides from hen ovalbumin (and some derivatives) which are shown in Figure 1. Structures 4–7 of Figure 1 occur commonly in side chains of glycoproteins.[2]

Results and Discussion

Figure 2 shows the spectra of 1, 4, 5, 6, and 7 (Structures of Figure 1). We did not isolate compounds 2 and 3, but we found them as transient products during the α-mannosidase digestion of 5 and 6.[3] We recorded ^{13}C NMR spectra during the α-mannosidase digestion and then we digitally subtracted the spectrum of free D-mannose in order to remove the resonances of the monosaccharide. In this fashion we were able to obtain the spectra of 2 and 3. Details have been published.[3]

We used a combination of the following information for assigning the ^{13}C resonances: (i) The chemical shifts of the oligosaccharides of the preceding paper.[1] (ii) Internal comparisons of the spectra of 1–7. (iii) Deuterium isotope effects on the chemical shifts of 1, 4, 5, and 7. (iv) The pH dependence of the chemical shifts of 1 and 4. (v) One-bond carbon–hydrogen scalar coupling constants of 4. (vi) Spin–lattice relaxation times of the ^{13}C resonances of 5.

We shall refer to the carbohydrate residues by their designations in Figure 1. A number following a residue designation indicates

(1) Allerhand, A.; Berman, E. *J. Am. Chem. Soc.* **1984**, *106*, preceding paper in this issue.
(2) Montreuil, J. *Adv. Carbohydr. Chem. Biochem.* **1980**, *37*, 157–223.
(3) Berman, E.; Allerhand, A. *J. Biol. Chem.* **1981**, *256*, 6657–6662.

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